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Role of Systemic Inflammation in Parkinson's Disease.

Systémový zánět v patogenezi Parkinsonovy choroby.

Diploma thesis

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My parents

To my hairy heroes

ABSTRACT

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Title of diploma thesis: Role of Systemic Inflammation in Parkinson's Disease.

Parkinson's disease (PD) is the second most common ageing-related neurodegenerative disorder after Alzheimer's disease and the prevalence in population is increasing. The characteristic movement disorder is caused by selective dopaminergic neurons loss, while the mechanism of this neurodegeneration is not well understood. Increasing evidence points out the key role of vicious cycle of microglial overactivation and oxidative stress, while the questions "where it begins" and „how to stop it" remain without clear answers. This thesis investigates implication of peripheral inflammation as a deteriorating circumstance and possible inductor of brain inflammation and progressive dopaminergic neurodegeneration. We use mice model of Parkinson's disease employing single intraperitoneal injection of toxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that induces specific degeneration of DA neurons and in contrast to other animal models, does not alter the integrity of blood-brain barrier (BBB) that is important in transmission of the peripheral inflammation to brain. Systemic inflammation is induced by intraperitoneal injection of lipopolysaccharide (LPS).

Our results show that peripheral inflammation triggers inflammation in brain, increases the activation of microglia and accelerate its onset, and exacerbates the dopaminergic neurodegeneration in substantia nigra caused by MPTP; all in absence of other BBB deleterious insult. Microglial activation appears earlier than loss of dopaminergic neurons, showing that overactivation of microglia is rather the cause than a consequence of neuronal death.

ABSTRAKT

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Kandidát: Karolína Veselá
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Název diplomové práce: Systémový zánět v patogenezi Parkinsonovy choroby.

Parkinsonova choroba (PD) je po Alzheimerově chorobě druhé nejčastější neurodegenerativní onemocnění, a s rostoucím průměrným věkem populace prevalence stále roste. Charakteristické příznaky jsou způsobeny úbytkem nervového přenosu v dopaminergní nigrostriatální dráze, přičemž patogeneze této neurodegenerace není doposud objasněna. Rostoucí poznatky ukazují, že klíčovým mechanismem je bludný kruh oxidativního stresu a patologické aktivace mikroglií, avšak otázky, kde tento proces začíná a jak ho zastavit, zůstávají bez jasných odpovědí.

Tato práce se zabývá rolí systémového zánětu jako faktoru zhoršujícího neurodegeneraci a jednou z možných příčin vyvolávajících chronický zánět v mozku spojený s úbytkem dopaminergních neuronů. Používá model intraperitoneálního podání neurotoxinu 1-methyl-4-fenyl-1,2,3,6-tetrahydropyridinu (MPTP) myším, který navozuje specifickou dopaminergní neurodegeneraci, a na rozdíl od ostatních modelů PD nezpůsobuje narušení hematoencefalické bariéry, jejíž (dys)funkce je důležitým faktorem při přenosu periferního zánětu do mozku. Systémový zánět je navozován intraperitoneálním podáním lipopolysacharidu (LPS).

Naše výsledky ukazují, že systémový zánět sám o sobě vyvolává zánět v mozku, zrychluje a prohlubuje aktivaci mikroglií vyvolanou MPTP a zvyšuje dlouhodobý úbytek dopaminergních neuronů vyvolaný MPTP. Mikrogliální aktivace nastává podstatně dříve než neurodegenerace, což potvrzuje její roli jako příčiny, a ne důsledku smrti neuronů.

CONTENTS

"The advantages which have been derived from the caution with which hypothetical statements are admitted, are in no instance more obvious than in those sciences which more particularly belong to the healing art."

James Parkinson, An Essay on the Shaking Palsy

1 INTRODUCTION

1.1 Parkinson's disease

1.1.1 Clinical and pathological features

The Parkinson's disease (PD) accompanies the humanity since its beginnings. It was described in Ayurveda books, as well as in Egyptian papyruses or Bible. Nowadays it is the second most common aging-related neurodegenerative disorder after Alzheimer's disease and the number of affected people is growing with the increasing average age of the population. The prevalence is approximately 1% of the population at the age of 55 and increases to 5% by the age of 85 (Block *et al*. 2007). PD is clinically described as movement disorder called "parkinsonism", which is demonstrated by resting tremor, muscle rigidity and akinesia. This is caused by deficit of dopamine (DA) in the striatum, result of progressive degeneration of dopaminergic neurons in the substancia nigra (SN). The mechanisms of this process are not clear.

The pathology of PD is also connected with identification of intraneuronal proteinaceous deposits, called Lewy bodies (LBs), and neuronal cytoplasmic inclusions, termed Lewy neurits (LNs) (Greenfield and Bosanquet 1953, Burguillos 2009). Their main component is aggregated form of α-synuclein, normally presynaptic protein (Braak *et al.* 2003). LBs and LNs are not specific for PD. They are also found in Alzheimer disease (AD) with LB, as well as in pure LB disease. They do not appear only in SN, but are detectable in various brain regions, such as locus coeruleus, raphe, thalamus, amygdala and cerebral cortex.

Braak, H. et al. divide the process of their distribution within the brain in six stages, with first of them describing the occurrence of LBs and LNs in the dorsal motor nucleus of the vagal nerve, and the last stage with nearly entire cortex involved (Braak *et al.* 2003). These findings indicate that PD can be involved in other neuronal system, not only dopaminergic (Chung *et al.* 2010).

More than 90% of the cases of PD are diagnosed as sporadic (Venero *et al.* 2011). Hereditary forms are associated with mutations of genes encoding several proteins, such as α-synuclein, leucin-rich repeat kinase 2 (LRRK-2), parkin, PTEN-induced kinase 1 and oncogen DJ1 (Farrer *et al.* 2006). However, experiments with animals carrying mutations or lacking some of PD-related genes show that abnormal functioning of these genes is rarely enough to cause parkinsonism (Jeong *et al.* 2010). For example parkin knock-out mice do not display SN neurodegeneration, but these mice suffered loss of DA neurons in SN after low-dose lipopolysaccharide (LPS) intraperitoneal administration, giving evidence of connection between this autosomal recessive form of PD and neuroinflammation (Frank-Cannon *et al.* 2008).

The symptoms of PD obviously manifest when the dopamine levels in striatum decrease to 20%, which corresponds to more than 50% loss of the dopaminergic cells in SN. The ability of the dopaminergic system to stand this huge loss without appearance of the PD symptoms is caused by compensatory mechanisms (Deumens *et al.* 2002).

To understand better the process of neurodegeneration, we need to search for its causes. Recently the attention has focused in mechanisms like mitochondrial dysfunction, oxidative stress, excitotoxicity and inflammation. These mechanisms are definitely related to eachother and may influence or potentiate their effect.

1.1.2 Neuroinflammation

The inflammatory component of the disease was suggested by some cases of PD associated to head trauma or encephalitis and studies demonstrating higher levels of pro-inflammatory cytokines as interleukin-1 (IL-1), interleukin-6 (IL-6), interferon gamma (IFNγ), cyclooxygenase 2 (COX2) and tumour necrosis factor alpha (TNFα), and presence of T-lymphocytes and reactive microglia in post mortem parkinsonian brains (Burguillos 2009, Venero *et al.* 2011, Herrera *et al.* 2000, Langston *et al.* 1999).

Some cohort studies on non-steroidal anti-inflammatory drugs (NSAIDs) reported decreased risk of PD in chronic users of these drugs (Chen *et al*. 2005). Further

investigations referred that only some of NSAIDs, such as ibuprofen, have this potential (Chen *et al.* 2003). However, results of clinical trials on treating neurodegenerative diseases with NSAIDs were more or less negative. Their limited effect can be derived from the fact that NSAIDs such as cyclooxygenase-2 (COX-2) inhibitors capture only prostaglandin E_2 (PGE_2), whereas the number of pro-inflammatory factors released from overactivated microglia is huge (Block *et al*. 2007).

Neuroinflammation is not specific only for Parkinson's disease, but is implicated in the neurodegeneration of Alzhaimer's disease as well as Huntington's disease, amyotrophic lateral sclerosis and other neurodegenerative diseases.

The factors playing important parts in DA neuronal death are microglial activation, reactive astrocytes, damaged blood-brain barrier (BBB) and infiltrated peripheral cells (Chung *et al.* 2010).

1.1.2.1 Microglial activation

Beside astrocytes, oligodendrocites and NG2 glial cells, microglia is one of the subtype of neuroglial cells. As a distinct cell entity they were first described by Pio del Rio-Hortega in 1919. They are the immune cells of CNS, housekeepers and guardians of the brain. Their origin is in circulating blood monocytes. In early embryonic development of the mammalians they populate the brain and adopt a ramified morphology. In adult individuals, microglia are located in all the regions of the brain and spinal cord and form 10-15% of all CNS cells (Venero *et al.* 2011, Chung *et al.* 2010).

Under physiological conditions, microglia serve as surveyors of the integrity and function of the brain. Non-activated microglia display ramified morphology. To a stimulus as damaged or apoptotic cell, antigens from infectious agents, DNA fragment or pathologically modified CNS proteins, microglia undergo activation, adopt a plastic ameboid morphology and phagocytize the undesirable material. Microglia also promote collateral sprouting, by releasing low levels of growth factors. By fulfilling these different functions, microglia play an important

homeostatic role in the CNS (Chung *et al.* 2010, Venero *et al.* 2011, Hald *et al*. 2005).

Activated microglia increase their gene expression, leading to production of pro-inflammatory mediators such as IL-1, TNFα, reactive oxygen/nitrogen species and proteases, and present diverse cell-surface receptors, such as complement receptors major histocompatibility complex molecules, chemokine receptors and other markers.

In chronic neurodegenerative diseases, microglia remain activated for a prolonged period and these potentially neurotoxic substances sustain longer than normal (Venero *et al.* 2011, Hald et al. 2005). The overactivation and disregulation of microglia might result in progressive neurotoxic consequences. In PD, the activated microglia is not only found in substancia nigra, but as well in putamen, hippocampus, transentorhinal cortex, cingulated cortex and temporal cortex (Block *et al*. 2007). The condition when activated microglia is present in large numbers is termed as "microgliosis".

The mechanisms leading to neurotoxic activation of microglia remain not well understood. Possible factors are neuronal damage, protein aggregates, and some environmental factors, such as toxins or pollutants (Venero *et al.* 2011).

Damaged DA neurons release factors such as matrix metalloproteinase 3 (MMP3), α-synuclein or neuromelanin, substances that activate microglia (Block *et al*. 2007). A study on MMP3 knockout mice pointed that DA neuron loss and microglial activation were lower in mice lacking this proteinase (Kim and Choi *et al.* 2007). Thus, microglia can be chronically activated by dying or damaged neurons leading to a vicious cycle of neuronal death and inflammation **(**McGeer *et al*. 2003).

Protein aggregates are involved in pathogenesis of PD and AD. In PD, α-synuclein is often surrounded by activated microglia. The relation with inflammation was observed in several studies on transgenic animals, for example mouse model overexpressing double-mutant human α-synuclein showed early microglia-mediated neuroinflammation before the neuron death (Su *et al*. 2009), and in other study, injection of inflammagen LPS within substantia nigra caused

neuroinflammation associated with accumulation of insoluble aggregated α-synuclein (Gao *et al*. 2008).

In conclusion, physiologic activation of microglia is essential for host defense of CNS, but overactivated microglia play important roles in the pathogenesis of neurodegenerative diseases.

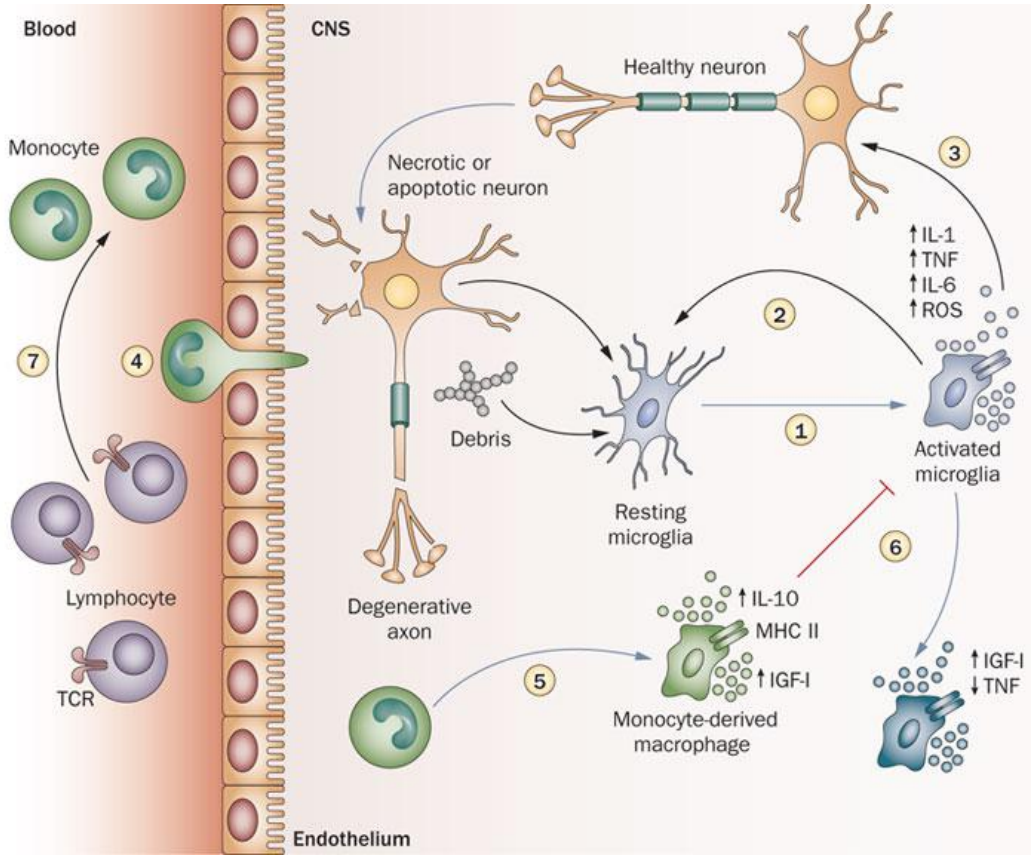

Figure 1. Immune cells in neurodegenerative conditions.
Dying cells, debris or aggregated proteins activate resting microglia in the CNS (1). Activated microglia phagocytose debris and aggregated proteins and secrete pro-inflammatory cytokines and ROS. If the microglial response is uncontrolled or prolonged, these toxic agents contribute to the development of a self-sustaining neurotoxic cycle that involves further activation of naive microglia (2) and loss of surrounding neurons (3). Blood-derived monocytes infiltrate the damaged parenchyma (4) and differentiate locally into macrophages (5). These cells efficiently engulf debris, secrete growth factors and anti-inflammatory cytokines, and suppress microglial activation (6). Under neurodegenerative conditions, resident microglia are unable to provide these essential functions. T-helper lymphocytes augment the recruitment of blood monocytes to the CNS (7). (Schwartz, M.; Shechter, R. Systemic inflammatory cells fight off neurodegenerative disease. *Nature Reviews Neurology*, 2010, 6, 405-410)

1.1.2.2 Reactive astrocytes

Astrocytes form 20-50% of brain volume. In the past they were considered to be non-excitable support cells of the brain, nowadays, they are seen as local communication cells. They modulate synaptic transmission, control the cerebral blood flow, and are also involved in the stimulation of postsynaptic neuronal excitability. They are as well involved in many neurological diseases (Volterra and Meldolesi 2005).

In contrary to their normal star-shaped morphology, reactive astrocytes have enlarged bodies and thick dendrites.

There are reactive astrocytes present in the SNc of PD patients, their role in the development of PD, however, is unexplained and controversial.

Under physiological conditions, astrocytes secret various neurotrophic factors. Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) have been shown to promote the survival of mesencephalic dopaminergic neurons in 6-OHDA and MPTP animal models of PD. In the pars compacta of substantia nigra (SNc) of post mortem PD brains, immunohistochemical and biochemical analyses showed decreased levels of these factors. Altough the role of BDNF is controversial (SNc pigmented neurons not showing BDNF had a greater probability of surviving than BDNF-positive pigmented neurons), GDNF has clearly proved its protecting action, and its decreased level is considered to be one of the early events anticipating neuronal loss in SNc in PD (Siegel and Chauhan, 2000).

Concluding, decreased levels of astroglia-derived neurotrophic factors are somehow connected to DA neuronal death in PD, although the relation between expression of the neurotrophic factors and reactive astrocytes is unknown (Chung *et al.* 2010).

Another important role of astrocytes is in antioxidant defense of the brain. Under oxidative stress conditions, nuclear factor erythroid 2-related factor 2 (Nrf2) binds to the antioxidant response element (ARE), which reduces production of reactive oxidative species (ROS). Jakel *et al.* (2007) showed that loss of Nrf2-mediated transcription can exacerbate vulnerability to 6-OHDA both in vitro and in vivo

and induction of Nrf2-ARE pathway by transplantation of astrocytes overexpressing Nrf2 can protect against 6-OHDA-induced damage (Jakel *et al*. 2007).

1.1.2.3 Blood brain barrier

Blood brain barrier (BBB) is a unique biological structure, that forms strict separation between blood and neural milieu, ensuring maintenance of narrow ranges of ionic concentrations, supply of nutrients to CNS cells, and protection from substances, that can be harmful to the CNS. It is composed of microvascular endothelial cells, astrocytes, basement membrane, and pericytes and neurons that are in physical proximity to the endothelium. Transport across the BBB is limited by tight junctions (composed of the integral transmembranous proteins, occludin, claudins, and junctional adhesion molecules), as well as enzymes and diverse transport systems.

Impaired function of BBB is present in various neurodegenerative and inflammation-related diseases of the brain, while the BBB disruption or alterations in transportation systems are often mediated by proinflammatory substances and pathogenic proteins, associated with the disease (Popescu *et al.* 2009, Persidsky *et al*. 2006).

BBB dysfunction is associated with aging. Several studies on aged rats suggested oxidative stress in brain parenchyma as a possible inductor of BBB alteration, associated with cell death, gliosis and signalling changes. Increased permeability of BBB can let abnormal molecules enter the brain and therefore induce microglia activation. In turn free radicals released from activated microglia can further damage the BBB, which can lead to a self-perpetuating cycle (Popescu *et al.* 2009). There is also evidence of increased transport of some substances across BBB of aged individuals, for example increased entering of blood-born TNFα into midbrain and striatum of aged mice brain was observed (Banks *et al.* 2001). Increased BBB permeability was further observed in MPTP and LPS models of PD (Chung *et al.* 2010).

However, not only increased permeability of the BBB, but also faulty of other neurovascular functions, such as clearance of toxic molecules, enzymatic function and nutrient supply are changes observed in aged and diseased brains. There is no evidence that these altered functions can directly cause loss of DA neurons in PD but several studies on PD patients and animal models suggest connection between BBB disruption and DA neuronal death.

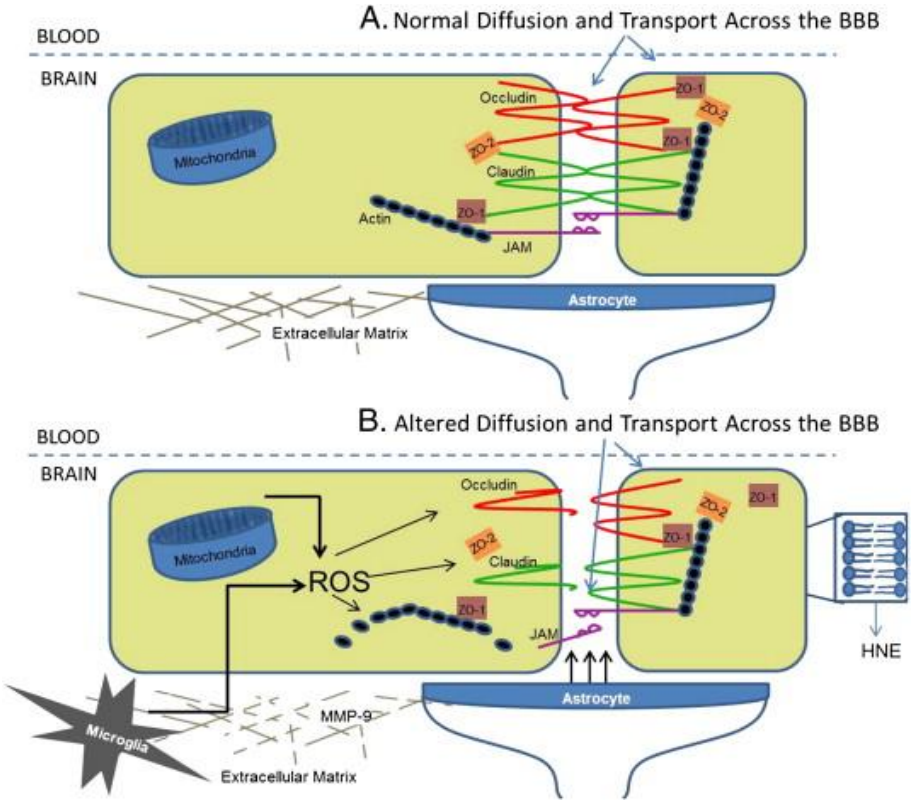

Figure 2. Diffusion and transport across the BBB.

In the healthy brain, the BBB is intact with the help of claudins, occludin, and JAMs to create the tight junction between endothelial cells. Actin and the zona occludens also create a scaffold for the tight junction. Further support for the BBB comes from the extracellular matrix and astrocytic end feet. In the unhealthy brain, ROS accumulate from various sources including mitochondria, microglia, and astrocytes. This causes altered assembly of the tight junctions, breakdown of the extracellular matrix by MMPs and subsequent loss of BBB integrity. (Freeman, L. R.; Keller, J. L. Oxidative stress and cerebral endothelial cells: Regulation of the blood–brain-barrier and antioxidant based interventions. *Biochimica et Biophysica Acta-Molecular Basis of Disease*, 2012, 1822, 822–829)

The first evidence supporting a dysfunctional blood–brain barrier as a causative mechanism in PD comes from positron emission tomography (PET) study on reduced function of p-glycoprotein pump in PD (Kortekaas *et al.* 2005). Some histological studies on PD patients revealed alterations of blood vessels, for example Faucheaux *et al.* (1999) pointed higher number of endothelial cell nuclei in SNc of patients with PD, comparing to aged-matched controls. Interestingly, levels of vascular endothelial growth factor (VEGF), and pigment epithelium-derived factor that induce structural changes in blood vessels were higher in PD patients, as well as in MPTP model (Chung *et al.* 2010). Moreover, correlation between BBB disruption and loss of tyrosine hydroxylase-positive neurons was proved in a study with VEGF injected rats (Rite *et al.* 2007).

1.1.2.4 Infiltration of peripheral cells

The presence of T-lymphocytes in the SN of PD patients suggests a role of infiltrated peripheral immune cells in the pathogenesis of PD.

In the LPS model of brain inflammation in normal and leukopenic rats injected with LPS into SNc, cortex or hippocampus, infiltrated monocytes were found in normal rats 24 hours after the administration (Ji *et al.* 2007). Other study showed resistance to MPTP in CD4 - defficient mice, suggesting an important role of T lymphocytes in neuroinflammation. Presence of ionized calcium binding adaptor molecule 1 (Iba-1, protein specifically expressed in macrophages and microglia, upregulated during the activation of these cells) positive cells in disrupted blood vessels provides a link between infiltration of peripheral immune cells and dysfunction of the BBB in SN (Chung *et al.* 2010).

1.1.3 Oxidative stress

Oxidative stress is caused by imbalance between production and destruction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can lead to cellular damage and death as a result of the modification of nucleic acids,

lipids and proteins (Chung *et al.* 2010). Reactive microglia upregulate various enzymes involved in the inflammatory process mediated by oxidative stress, such as inducible NO synthase (iNOS), NADPH oxidase, cyclooxygenase-2 (COX-2) and myeloperoxidase. Generation of ROS by activated microglia through the NADPH oxidase system, is an important antimicrobial mechanism, but in the condition of neurodegenerative disease it can be the most critical factor inducing the DA neuronal death (Venero *et al.* 2011, Qin *et al.* 2004). ROS produced at normal physiological conditions are scavenged by endogenous antioxidants including glutathione peroxidase, superoxide dismutase, catalase and vitamin C and E, but these protective factors are impaired in diseased brain. In a healthy brain, carbonyl modifications, that are indicative of protein oxidation, are increased 2-fold in the SN compared to basal ganglia and prefrontal cortex (Chung *et al.* 2010). Thus, physiologically increased intracellular oxidative process is a normal characteristic of dopaminergic neurons, making them particularly vulnerable to oxidative stress (Whitton 2007). This feature is related to the metabolism of dopamine (Qin *et al.* 2004).

There are two ways of degradation of dopamine. First of them is metabolism by monoamine oxidase (MAO) that leads to the production of dihydrophenylacetic acid (DOPAC) and H_2O_2 under consumption of O_2 and H_2O. Second is autooxidation, which generates H_2O_2 and dopamine-quinone. In the presence of ferrous iron (Fe^{2+}), H_2O_2 can be converted into hydroxyl radicals that react with almost all macromolecules and cause widespread damage. This process can be important in pathogenesis of PD, given that iron levels in SN are higher than in other areas of the brain, and in PD patients are increased by 35%, compared to age-matched controls. Dopamine-quinone then participates in nucleophilic adition reactions with protein sulphuryl groups, leading to structural modifications of proteins and reduced levels of glutathione, the major cellular redox buffer, important for overcoming the oxidative stress (Chung *et al.* 2010). Decreased levels of reduced glutathione were observed in SN of PD patients compared to controls (Sofic *et al.* 1992). Dopamine or its oxidized metabolites can also interfere with complex I and inhibit mitochondrial respiration (Ben-schachar *et al*. 2004).

In the MPTP model of PD, NADPH oxidase and iNOS are major sources of ROS/RNS production, leading to DA neurodegeneration via oxidative stress (Chung *et al.* 2010). The important role of these enzymes is supported by studies showing protection against LPS induced neuroinflammation in NADPH deficient mice or pharmacologically inhibited NOS (Chung *et al.* 2010, Venero *et al.* 2011, Qin *et al.* 2004). Neurodegeneration in mice lacking NADPH oxidase was also less strong in an experiment with exposure to α-synuclein (this protein has greater vulnerability to aggregate when oxidized, moreover phagocytized by microglia provokes activation of NADPH oxidase and ROS production) (Hald *et al.* 2005, Glass *et al.* 2010).

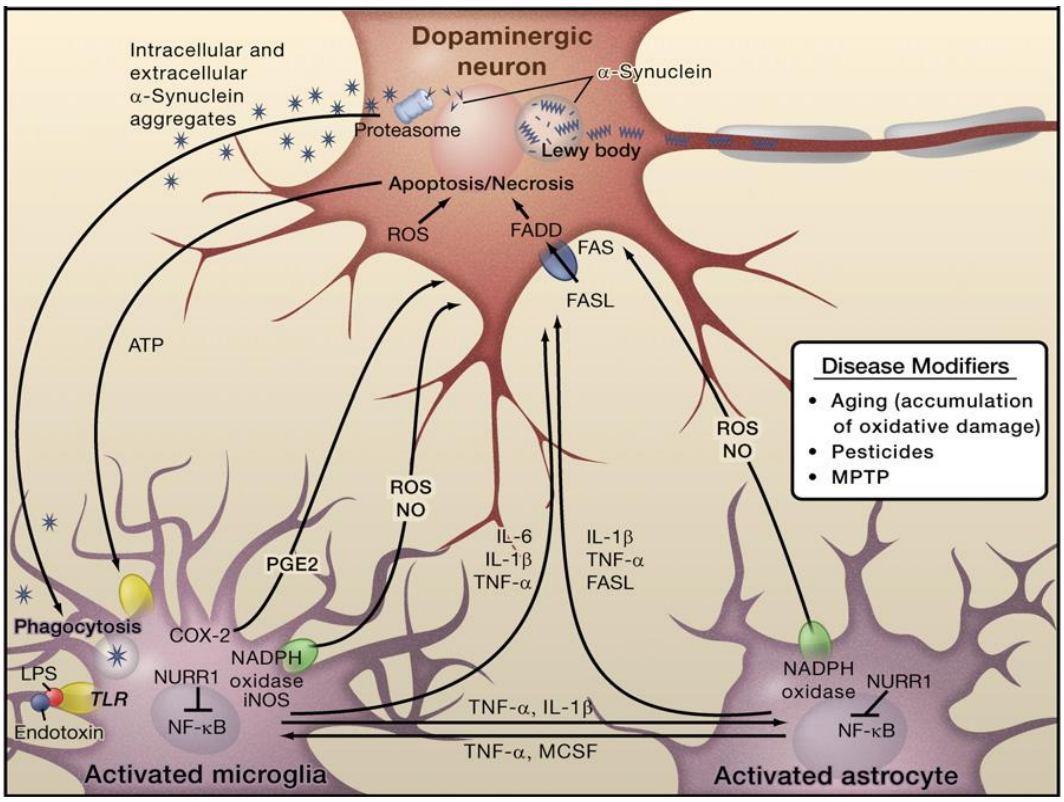

Figure 3. Inflammatory components in Parkinson's disease.
Besides forming Lewy bodies, aggregates of α-synuclein form intermediate-state oligomers that, when released from neurons, activate microglia through TLR-independent mechanisms. This leads to activation of NF-κB and production of ROS and proinflammatory mediators. These factors act directly on dopaminergic neurons of the substantia nigra and activate microglia, which amplify the inflammatory response in a positive feedback loop, leading to further activation of microglia. **(**Glass, C. K. et al. Mechanisms Underlying Inflammation in Neurodegeneration. *Cell*, 2010, 140, 918–934)

1.1.4 Role of systemic inflammation

The fact that systemic inflammation communicates with our brains is clear, thanks to our experiences with changes in mood, emotion and cognition associated with common systemic infections. These metabolic and behavioral changes help us to fight the infection, namely febrile response inhibits proliferation of pathogens, behavioral changes serve to conserve energy and avoid transmission of the disease to other individuals, and anorectic response prevents pathogens from obtaining essential nutrients (Perry 2010).

The signaling from inflammatory periphery to the brain goes through both neuronal and humoral routes. For instance, during inflammation of thoracic abdominal cavity, signaling to brain goes via vagal afferent fibers, which terminate in nucleus of the solitary tract, which then stimulates the regions of the brain involved in motivation and mood. Signaling via efferent fibers then secrete acetylcholine, which, acting on nicotinic receptors of macrofages, leads to downregulation of the secretion of inflammatory cytokines. Humoral pathway is realized by cytokines which enter the general circulation and communicate directly with macrophages and other cells in the BBB lacking circumventricular organs (the signal is then spread to CNS via microglia), or interact with the receptors on the brain endothelium (Perry *et al.* 2007). There is also possibility of directly entering the brain through disrupted BBB. Altough this cytokine-mediated communication has a great importance, as has been demonstrated in cytokine-deficient mice, there are more signaling routs from the peripheral immune system to the brain, while many of them has not been well clarified (Perry 2010).

There is increasing evidence about the effects of systemic inflammation on diseased CNS. About one-third of relapses of multiple sclerosis, an archetypal inflammatory disease of the CNS, are associated with systemic inflammation, typically upper respiratory tract infections (Perry 2010). Interestingly, clinical exacerbations of MS were not associated with BBB opening, which suggest some signaling across an intact BBB (Buljevac *et al*. 2002). In a case-control study on

AD, increased probability of diagnosis was found in people with more episodes of infections (Dunn *et al.* 2005). Inflammation is also known to be a risk factor for PD. Higher incidence of peptic ulcer in patients with idiopathic PD, comparing to controls, has been reported (Machado *et al.* 2011). Acute infection was found to increase the risk of stroke and worse outcome of stroke was described in patients presenting with antecedent infection (McColl *et al.* 2008). Interestingly, there is also evidence of cognitive impairments in elderly people as a result of peripheral inflammation (Godbout *et al.* 2005).

The above mentioned effect of NSAIDs on incidence of PD can be explained as inhibition of neuroinflammation, but can also be a result of peripheral inflammation inhibition.

Taken together, peripheral inflammation is able to enhance ongoing neurodegeneration or produce sensitivity to other neurodegenerative insults, and therefore increase the incidence of neurodegenerative diseases.

1.2 LPS

Lipopolysaccharide (LPS) is the main component of cell wall of gram-negative bacteria and is potent inductor of inflammation. Common structure of LPS consists of three parts. (1) O antigen, a repetitive glycan polymer, (2) core, divided in external core formed by hexoses and internal core formed by heptoses, and (3) lipid A, phosphorylated glucosamine disaccharide with fatty acids, responsible for the toxicity (Burguillos 2009, Wang and Quinn 2010).

It has been found that an intranigral injection of LPS causes strong microglial activation and degeneration of DA neurons in SN. The damage was still clearly evident 1 year after the injection, and was selective for dopaminergic neurons, as there was no measurable damage to GABAergic or serotonergic neurons at the same LPS doses. Therefore it was suggested as a model of PD (Herrera *et al.* 2000).

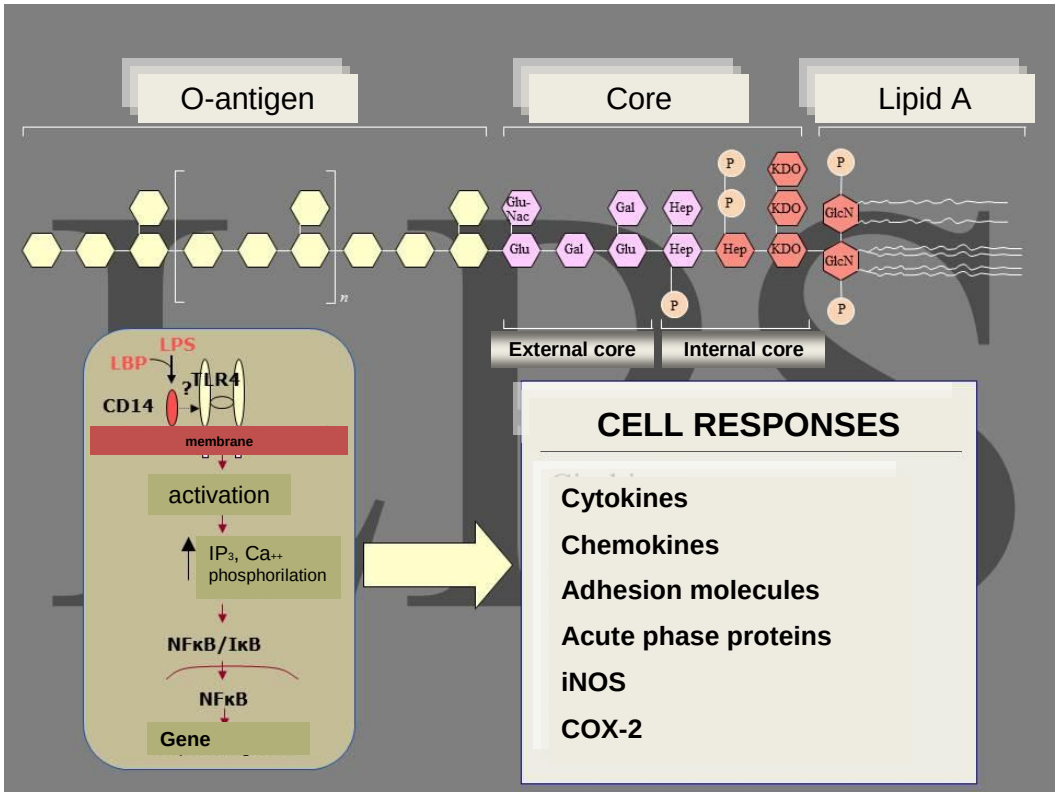

Figure 4. Structure and mechanism of action of LPS.

Injected to SN, LPS acts primarily through toll-like receptors (TLR) expressed on microglial cells, triggering nuclear localisation of transcription factor NFκB, and subsequent transcription of genes implicated in the proinflammatory pathways. However, there are also other pathways of LPS microglial activation. Of note, caspases 8, 3 and 7 have been demonstrated to play a significant role in activating NFκB trasnscription factor and subsequent microglia activation (Burguillos et al. 2011).

Microglia is much more responsive to LPS than astrocytes, whereas neurons are practically unresponsive (Glass *et al.* 2010). Thus, microglia are the primary initial responders to LPS, confirming the key role of inflammation in neurodegeneration.

Other effect of LPS is induction of expression of caspase-11. Caspase-11 knockout mice were found resistant to LPS-induced increases in IL-1β, microglial activation and DA cell loss (Whitton 2007). LPS can also synergize with mutations in α-synuclein and parkin to potentiate the loss of TH-positive neurons

in animal models (Glass *et al.* 2010). Dopamine itself can be involved in LPS-induced neurodegeneration. Decrease of DA synthesis as a consequence of TH inhibition by α-methyl-p-tyrosine had preventive effect on the degeneration of dopaminergic neurons (De Pablos *et al.* 2005).

LPS injected intraperitonealy, reaching bloodstream binds with serum proteins, LPS binding protein or septins, these complexes bind to CD14 receptors of monocytes/macrophages, inducing release of proinflammatory cytokines, which are considered directly responsible for the LPS inflammation response (Lacroix *et al*. 1998). Systemic LPS injection increases gene expression for TLR2, COX-2, IL-6 and IL-6 receptor in the CNS, causes breakdown of BBB and leads to infiltration of granulocytes into the CNS (Whitton 2007). Beside this, it induces changes in blood pressure, osmolarity, pain, oxygen consumption, energy metabolism, provokes fever, and causes various changes in the endocrine system (Lacroix *et al*. 1998).

1.3 MPTP

The effect of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on dopaminergic system was first recognized in an occurrence of irreversible symptoms and signs of parkinsonism among a group of heroin addicts, who self-administrated drugs contamined with this neurotoxin (Nussbaum *et al.* 1997, Langston *et al.* 2010).

The mechanism of its selective neurotoxicity is the following: After its systemic administration, MPTP, which is highly lipophilic, crosses the BBB and is converted in glial cells by monoamine oxidase B to ion ($MPDP^+$), and then, probably by spontaneous oxidation, is converted to 1-methyl-4-phenyl-pyridiniuym (MPP^+), which is the active neurotoxic substance. MPP^+ is substrate of active presynaptic dopamine uptake system (has high affinity for plasma membrane dopamine transporter). The concentration in neurons is energy-dependent, concentrated by active process within the mitochondria. MPP^+

accumulates in mitochondrial matrix against a concentration gradient, driven by electrochemical potential of the membrane. It impairs the mitochondrial respiration by inhibiting the complex I of the electron transport chain, which leads to rapid ATP depletion and increased production of free radicals, especially superoxide. Superoxide is not very reactive and cannot cause a serious direct damage but its toxicity is based on production of other reactive molecules, such as hydroxyl radical. The superoxide can as well react with NO, coming from neighboring cells, and form peroxynitrite that can damage lipids, provoke DNA modifications and strand breaks, and covalently modify tyrosine residues leading to a disruption of enzyme function and structural protein integrity. These events can lead to apoptosis or necrosis (Tipton and Singer 1993, Schapira 1995, Przeborski *et al.* 2000, Hald and Lotharius 2005).

Since the discovery that MPTP causes parkinsonism, it has been used as a model of PD. The effects of MPTP were further studied in humans and non-human primates, rats, mice and other mammalians (Przeborski *et al.* 2000, McGeer *et al*. 2003).

The damage to the nigrostriatal DA pathway includes features such as mitochondrial activation, oxidative stress and inflammation, it has some limitations, for example absence of Lewy bodies (Chung *et al.* 2010, Burguillos 2009).

In addition, that beside the direct effect of MPTP on the neurons, it is also considered that it acts indirectly by overactivating microglia. Its administration can lead to reactive microgliosis and vicious circle of neurodegeneration (Chung *et al.* 2010). This is supported by studies showing that the MPTP toxicity is reduced in mutant mice deficient in pro-inflammatory factors, such as TNFα, mieloperoxidase, NO or prostaglandins (Block *et al*., 2007).

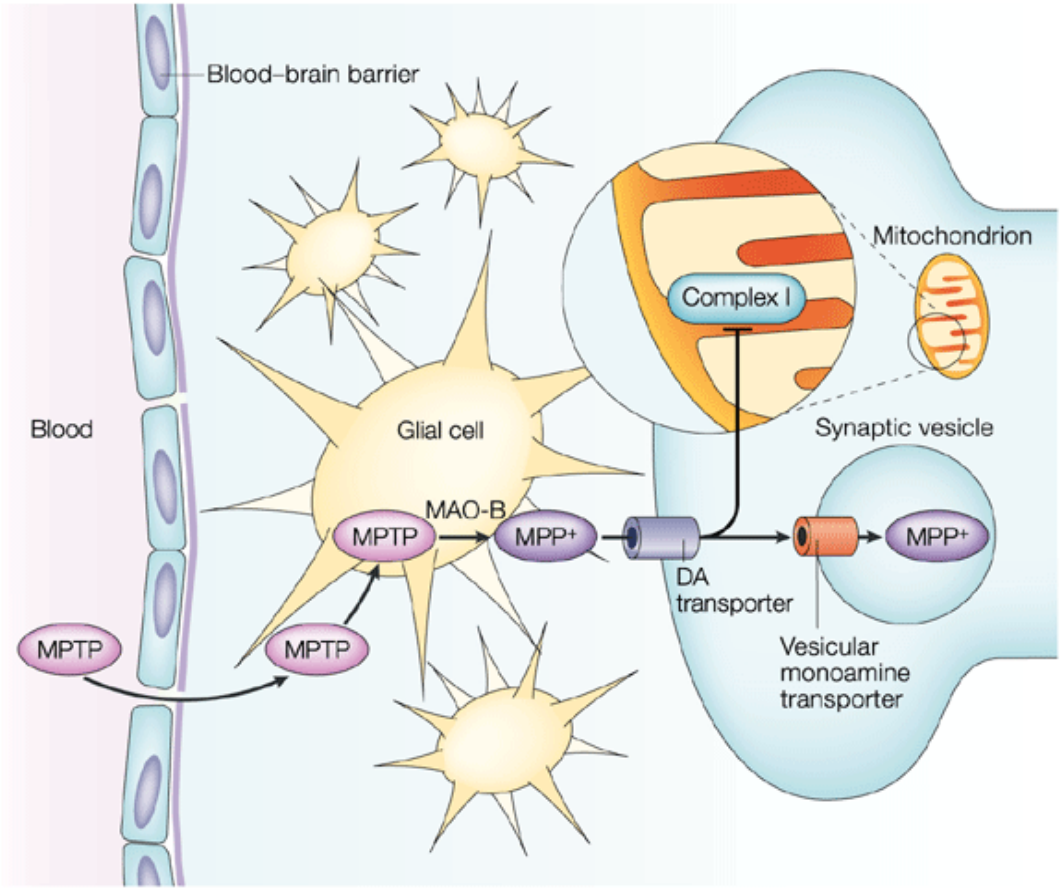

Figure 5. MPTP - mechanism of action.
After its systemic administration, MPTP, which is a pro-toxin, rapidly crosses the blood–brain barrier and is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by the enzyme monoamine oxidase B (MAO-B) in non-DA cells, and then, probably by spontaneous oxidation, to 1-methyl-4-phenylpyridinium (MPP+), the active toxic compound. MPP+ is then taken up by DA transporters, for which it has high affinity. Once inside DA neurons, MPP+ is concentrated by an active process within the mitochondria, where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain. The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, resulting in an increased production of free radicals, which causes oxidative stress and activation of programmed cell death molecular pathways. (Vila, M.; Przedborski, S. Targeting programmed cell death in neurodegenerative diseases. *Nature Reviews Neuroscience*, 2003, 4, 365-375)

2 AIMS

The main goal of this study was to test how acute peripheral inflammation influences the specific neurodegeneration of the nigro-striatal dopaminergic system and the inflammatory response in the MPTP animal model of PD. The animal model of PD exhibits some key features, including absence of damage to the BBB, which are critical for getting further insights into the real influence of peripheral inflammation in brain neurodegeneration.

Our goals are the following

a) to analyze if acute systemic inflammation is enough to trigger brain inflammation in brain areas typically related to PD in absence of other brain deleterious stimulus.

b) to analyze if acute systemic inflammation alters the time-course of changes of microglial activation in the nigrostriatal system in the MPTP animal model of PD. Special attention will be paid to temporal changes of microglia activation and dopaminergic degeneration

c) to analyze if acute inflammation further enhances the impairment of the nigro-striatal dopaminergic system in the MPTP animal model of PD. To achieve this, stereological quantification of tyrosine hydroxylase-immunopositive neurons will be performed in the ventral mesencephalon in response to MPTP alone or a combination of systemic LPS+MPTP.

3 MATERIALS AND METHODS

3.1 Animals and treatments.

Male mice (race C57BL/6) were used for these studies. They were housed in groups of 4-6, at constant room temperature of 22 ± 1 ºC and relative humidity (60%), with a 12-h light-dark cycle and free access to food and water.

Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU), following the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals and approved by the Scientific Committee of the University of Seville.

Animals were divided into groups according to the treatments, time points and method of analysis.

These groups are defined as follows:

Immunohistochemistry.

LPS 14 hours

LPS 24 hours

LPS 2 weeks

MPTP 12 hours

MPTP 24 hours

MPTP 2 weeks

LPS and MPTP 12 hours

LPS and MPTP 24 hours

LPS and MPTP 2 weeks

Control animals

qPCR

MPTP 12 hours

LPS and MPTP 12 hours

Control animals

HPLC

MPTP 2 weeks

LPS and MPTP 2 weeks.

Control animals

Animals received a single intraperitoneal injection of LPS (2 mg/kg in 0.9% sterile saline), single intraperitoneal injection of MPTP (40 mg/kg in 0.9% sterile saline) or both. In the case of using both treatments, LPS was administered 120 min before the MPTP administration. All the treatments were administrated in a volume of 100 µl/25g.

At required day and hour after the treatment, animals dedicated to the immunohistochemistry were anaesthetized with chloral hydrate (6 g/100 ml solution, 150-200 µl per animal) and perfused through the heart with 0.9% saline followed by 40 ml of 4% paraformaldehyde in phosphate buffer, pH 7.4. Brains were removed and then cryoprotected serially in sucrose in PBS, pH 7.4, first in 10% (24 hours), then in 20% sucrose (24 hours), and then in 30% sucrose until sunk (2-5 days). The brains were then frozen in isopentane at –15 ºC (10 minutes) and kept at –40 ºC.

Animals designated to HPLC and qPCR were sacrificed by decapitation at appropriate day and hour after the treatment, brains were removed and striatum and substancia nigra disected. It was snap-frozen in liquid nitrogen and kept at temperature of –80ºC.

Mortality caused by treatment

In the groups of MPTP treated mice a total number of 32 mice were injected with this drug. Seven mice died during the first 24 hours after the treatment. The survival was 78%.

Within the mice treated with both LPS and MPTP, a total number of 21 mice survived out of the total number of 39 mice injected with the drugs.
17 out of the 18 died mice did not survive the first 24 hours after the treatment. The survival was 54%.
In the group of animals treated with LPS, all the mice survived the required period, as well as within control animals, the survival was 100%.

3.2 Immunohistochemistry

25 μm coronal sections were cut on a cryostat at –20 ºC and mounted in gelatine-coated slides.

Primary antibodies used were:
rabbit-derived anti-tyrosine hydroxylase (Sigma, 1:300)
rabbit-derived anti-Iba1(Wako, 1:500)
rabbit-derived anti-glial fibrillary acidic protein (1:300)
rabbit-derived anti-cleaved caspase 8 (Cell Signalling, 1:150)

Incubations and washes for all the antibodies were performed in Tris-buffered saline (TBS), pH 7.4. All work was done at room temperature, except the incubation with primary antibody, which was done at temperature of 4 ºC.
Sections were defrosted and dried. The area around each section was demarcated with a hydrophobic pen (Dako Pen, x). Slides were then washed in TBS and heated in microwave with citrate buffer (500 kWh, 4 minutes twice), then washed with TBS (three 10 minutes washes) and treated for 15 min with 1% hydrogen peroxide in methanol, washed twice in TBS again, and incubated for 60 min in a humid chamber in a solution containing 1% goat serum in TBS.
Slides were drained and further incubated overnight with the primary antibody in TBS containing 1% goat serum and 0.25% Triton-X-100.
Next day, sections were washed in TBS (three 10 minutes rinses) and incubated for 2 hours with biotinylated goat anti-rabbit IgG (Vector, 1:200). The secondary antibody was diluted in TBS containing 0.25% Triton-X-100. After this step,

sections were rinsed again (three 10 minutes washes) and incubated for 1 hour with ABC-Peroxidase staining kit (Thermo, 1:101). The peroxidase was visualized with a standard diaminobenzidine/hydrogen reaction for 6 min.

3.3 Dehydration and mounting of the slides

The sections were dehydrated with ethanol in increasing concentrations (50%, 70%, 90% for 5 minutes in each and ten minutes in absolute ethanol) and impregned with histolemon (15 minutes). After that, DPX glue was used and sections were covered with cover glass.

3.4 Immunofluorescence

The primary antibody used was goat derived polyclonal anti-occludin (Santa Cruz, 1:100)

The immunofluorescence was performed on corpus striatum sections in the early time points' groups. Immediately after defrosting and drying the sections, the primary antibody (dilution in TBS containing 0.25% Triton-X-100) was added. The sections were incubated at 4ºC overnight. Three rinses with TBS were proceeded the next morning, and then the sections were incubated for 3 hours with the secondary antibody (anti-goat fluoresceing secondary antibody, Vector, diluted in TBS with 0.25% Triton-X-100). The slides were then washed in TBS again (three 10 minutes washes), mounted with Dako fluorescent mounting medium, covered with the cover glass and kept at 4 ºC. The slides were kept in dark during all the steps.

3.5 Analysis of the immunohistochemical data

The brains of MPTP treated mice, and LPS + MPTP treated ones, both groups sacrificed 14 days after the treatment, were cut at the cryostat, two at the same time, one from each group. Immunohistochemistry with TH antibody was performed. For the measurement of the number of the dopaminergic neurons, we used the AnalySIS imaging software (Soft Imaging System GmbH, Münster, Germany) coupled to a Polaroid DMC camera (Polaroid, Cambridge, MA) attached to a Leika light microscope (Leika Mikroskopie, Wetzlar, Germany). Cells showing TH immunoreactivity were counted by using five sections per animal systematically distributed through the SN anterior-posterior axis. In each section, a systematic sampling of the area, occupied by the TH positive cells, was made from a random starting point with a grid adjusted to count seven fields per section. An unbiased counting frame of known area (15686,7 μm^2) was superimposed on the tissue section image under a 40x objective. Therefore, the area sampling fraction was 15686,7μm^2 / 63380,6μm^2 = 0.248. In all animals, 25-μm sections, each 175 μm apart, were analyzed; thus, the fraction of sections sampled was 25/175 = 0.143. The total number of neurons in the SN was estimated by multiplying the number of neurons counted within the sample regions by the reciprocals of the area sampling fraction and the fraction of the sampled section.

3.6 HPLC analysis

Animals were sacrificed by decapitation and corpus striatum was dissected. It was deep frozen in liquid nitrogen and stored at -80 ºC.

The tissue was weighted and 0.1 M perchloric acid containing 1 mM sodium bisulfite (20 μl of per 1 mg of tissue) was added. The homogenization was done by ultrasonic disintegration over ice using Labsonic 1510. Samples were centrifuged at 12000 rpm for 5 minutes at 4 ºC. Supernatant was filtered by 0.2 μm filter.

Material was injected by high pressure injection valve (Rheodyne), with a 10µl sample loop.

Dopamine and its metabolites in striatal tissue were analysed by HPLC with electrochemical detection.

A Merck L-6200A intelligent pump was used in conjunction with a glassy carbon electrode set at +550 mV (DECADE II, ANTEC, The Netherlands). A Merck Lichrocart cartridge (125 x 4 mm) column filled with Lichrospher reverse-phase C_{18} 5 µm material was used.

The mobile phase consisted of a mixture of sodium acetate (0.05 M), 1-octanesulfonic acid (0.4 mM), Na_2EDTA (0.3 mM) and methanol (70 ml/l), and was adjusted to pH 4.1 with acetic acid.

Analyses were performed in the isocratic mode, at flow rate of 1.0 ml/min and at 30º C. The detection limit for dopamine was 5 fmol per injection.

The standard solution was prepared with solutions of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), serotonine, and 5-hydroxyindoleacetic acid (5-HIAA), all of them in concentration 10 ng/ml in the final solution.

All the reagents and water were HPLC grade.

3.7 RT-PCR

The striatum and substantia nigra samples were weighted and homogenized in RLT buffer with 1% β-merkaptoethanol with politron. RNeasy® kit (Qiagen) was used for the extraction and purification of total RNA. The concentration of the RNA was measured with NanoDrop ND 1000 spectrophotometer (DICSA). Genomic DNA elimination and reverse transcription was done using QuantiTect® reverse transcription kit (Qiagen). 500 ng of total RNA was used in 14 µl reaction volume, following the manufacturer protocol.

Real-time PCR was performed with SensiFAST™ SYBR No-ROX kit (Bioline), with concentration of primers 400 nM, and 500 ng cDNA. Amplification was run in LightCycler® 480 SW 1.5 (Roche) thermocycler. Briefly, cDNA was denatured for 2 min at 95 ºC, followed by 50 cycles of denaturation at 95 ºC for 5 s and

annealing/extension at 60 ºC for 15 s. After reaction completion, melting curve analysis (from 60 ºC to 95 ºC) was performed to verify specificity of the reactions. Relative quantification was calculated using LightCycler 480 software. G6PDH served as reference gene and was used for normalization of the samples.
Sequences for IL-6, TNF-α, iNOS, and G6PDH are as follows:

TNFα forward: 5'- AGCCCACGTCGTAGCAAACCACC
TNFα reverse: 5'- AACACCCATTCCCTTCACAGAGC

IL6 forward: 5'- TTCTTGGGACTGATGCTG
IL6 reverse: 5'- CTGGCTTTGTCTTTCTTGTT

iNOS forward: 5'- CTTTGCCACGGACGAGAC
iNOS reverse: 5'- TCATTGTACTCTGAGGGCTGAC

G6PDH forward: 5'- AACGACCCCTTCATTGACC
G6PDH reverse: 5'- TCAGATGCCTGCTTCACC

4 RESULTS

4.1 Expression of the cytokines

We measured the expression of mRNA encoding for IL-6, TNFα and iNOS in substantia nigra and striatum of mice sacrificed 24 hours after the intraperitoneal injection of MPTP in animals treated with MPTP, animals treated with both LPS and MPTP, and control animals with no treatment. In the case of animals treated with both substances, LPS was administered 2 hours prior the injection of MPTP. In all cases of treated animals, the expression of the cytokines was increased comparing to controls. The level of mRNA in SN and striatum in control animals was so low, that the cDNA has not been amplified within the first 50 cycles of RT-PCR (Figures 6, 7, 8).

Earlier amplification of cDNA obtained from animals undergoing systemic inflammation after intraperitoneal injection of LPS (LPS+MPTP treated animals), compared to animals treated with MPTP only, shows higher expression of IL-6 in their brains 24 hours after the treatment (Figure 6). The relative quantification results showed that IL-6 expression (Figure 9, green columns) was 3,4-fold increased in striatum of animals treated with both LPS and MPTP compared to animals treated with only MPTP, and 1,8 fold increased in their SN.

The mRNA encoding for TNFα expression was increased in animals treated with MPTP as well as in animals treated with LPS+MPTP (Figure 7). The relative quantification shows that expression of mRNA encoding for TNFα (Figure 9, red columns) was increased in SN of animals that received both treatments comparing to animals receiving only MPTP, but interestingly, at this time point in striatum the expression was not higher in animals receiving LPS.

Similarly the amplification curves indicated that the expression of iNOS was higher in treated compared to control animals (Figure 8). Relative quantification showed that 24 hours post injection the expression of iNOS was lower in animals treated with LPS and MPTP, comparing to animals treated with MPTP only, both in SN and striatum (Figure 9, blue columns).

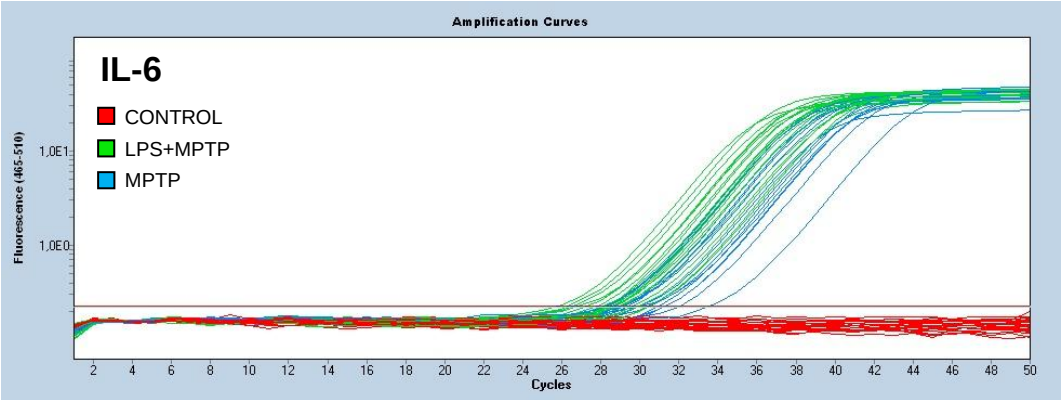

Figure 6. Effect of systemic MPTP and LPS on mRNA expression of IL-6 in substantia nigra and striatum. The red lines show amplification curves of samples of cDNA from control animals. Blue curves indicate expression of mRNA encoding for IL-6 in animals treated with MPTP and green colour refers to animals treated with both LPS and MPTP.

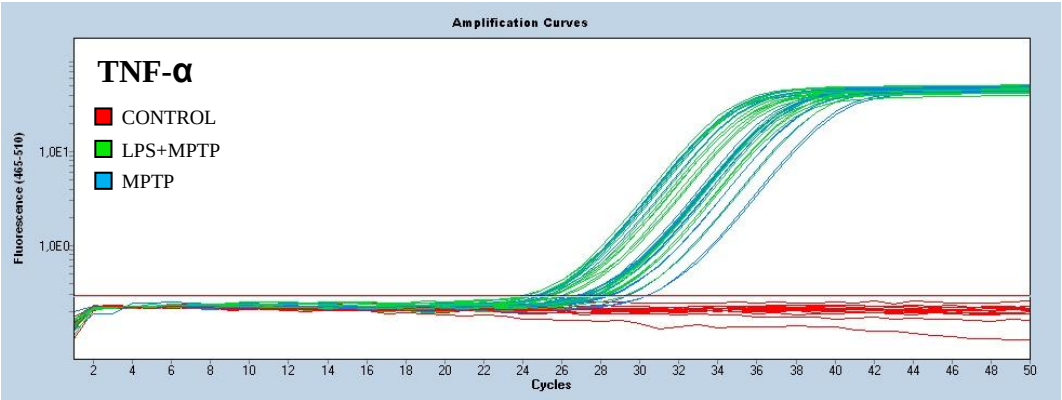

Figure 7. Effect of systemic MPTP and LPS on m RNA expression of TNFα in substantia nigra and striatum. The red colour refers to amplification curves of control animals, which have not crossed the noise band. TNFα expression was higher in animls treated with MPTP (blue colour) and animals treated with both substances (green).

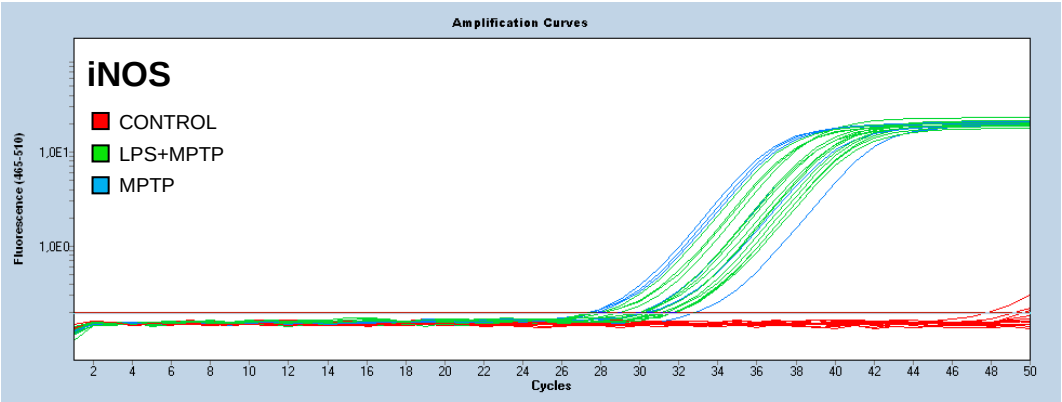

Figure 8. Effect of systemic MPTP and LPS on mRNA expression of iNOS in substantia nigra and striatum. mRNA encoding for iNOS has not been detected in control animals (red colour). In animals treated with LPS and MPTP, the expression was higher, as the cDNA in samples from these animals was amplified.

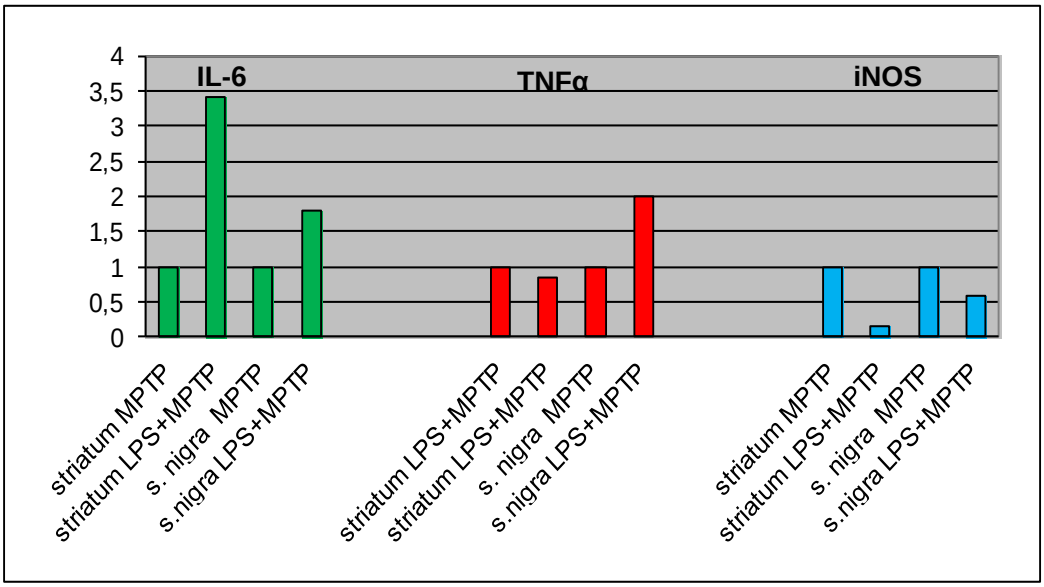

Figure 9. Relative quantification of expression of cytokines in SN and striatum of animals treated with LPS and MPTP. The expression of IL-6, TNFα, and iNOS was measured by RT-PCR 24 hours after the injection of MPTP (in group of animals recieving both treatment, LPS was injected 2 hours prior to the injection of MPTP).

4.2 Microglia

4.2.1 Striatum

Control animals showed faint Iba1 immunostaining, with small microglial cells. Treatment with LPS-alone induced a transient activation that peaked at 12h. Microglial bodies were bigger and the density of the visible cells was 51% higher than control at 12h and 43% higher at 24h after the treatment (Figures 10, 11; b, e). The treatment with MPTP showed a time course characterized by a slight activation at 12 h to further increase at 24 h (Figures 10, 11; c, f). 12 hours after the treatment the density and visibility of the cells increased comparing to control (121%), but this was less than in LPS 12h. 24h post injection the density was comparable to MPTP 24h (154% compared to controls). The most significant changes were seen in LPS+MPTP-treated animals. Under these conditions, strong activation was observed at 12 h after the injection of MPTP. This activation was significantly higher when compared with either LPS- or MPTP-injected animals (213% compared to control). The high microglial activation seen in LPS+MPTP-injected animals was still evident 24 hours after the treatment. The activated cells showed typical morphology with shorter and thicker processes and increased Iba1 immunostaining (Figures 10, 11; d, g).

2 weeks after treatment, the morphology of most of the microglial cells was similar to control, although some cells in the striata of LPS+MPTP as well as in MPTP treated animals showed a round, phagocytic morphology without processes, typical for chronically microglia (Figure 11; i, j). In the animals treated only with LPS, there were no clear observations of phagocytic microglia (Figure 11, h).

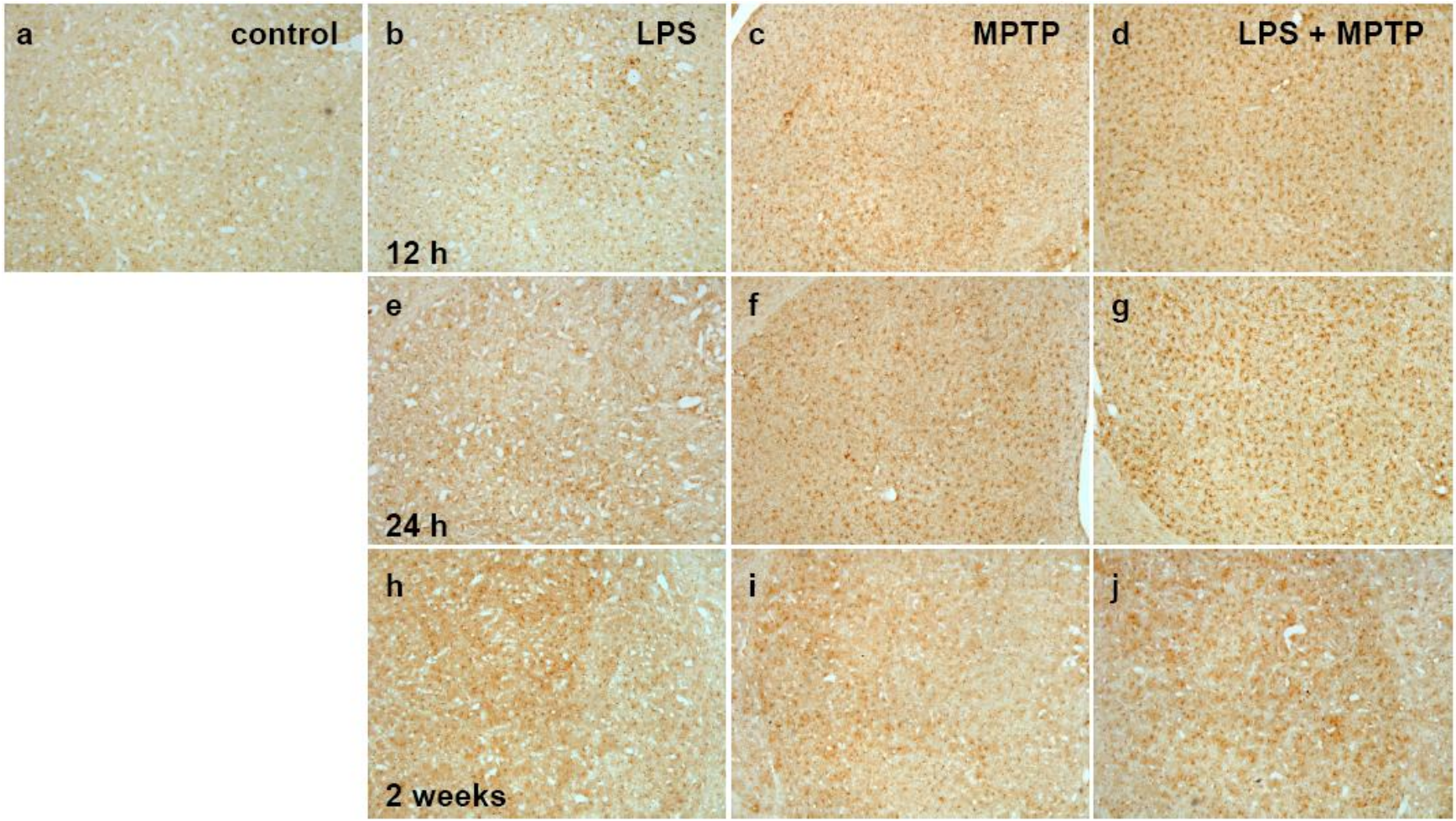

Figure 10. Effect of systemic LPS on MPTP-induced microglia activation in the striatum. Figure shows low magnification (5x lens) photographs of Iba1 immunohistochemistry in the substantia nigra. (a) Control, untreated animals. (b) LPS, 12h. (c) MPTP 12h. (d) LPS + MPTP 12h. (e) LPS 24h. (f) MPTP, 24 h. (g) LPS+MPTP, 24h. (h) LPS, 2 weeks. (i) MPTP, 2 weeks. (j) LPS + MPTP 2 weeks.

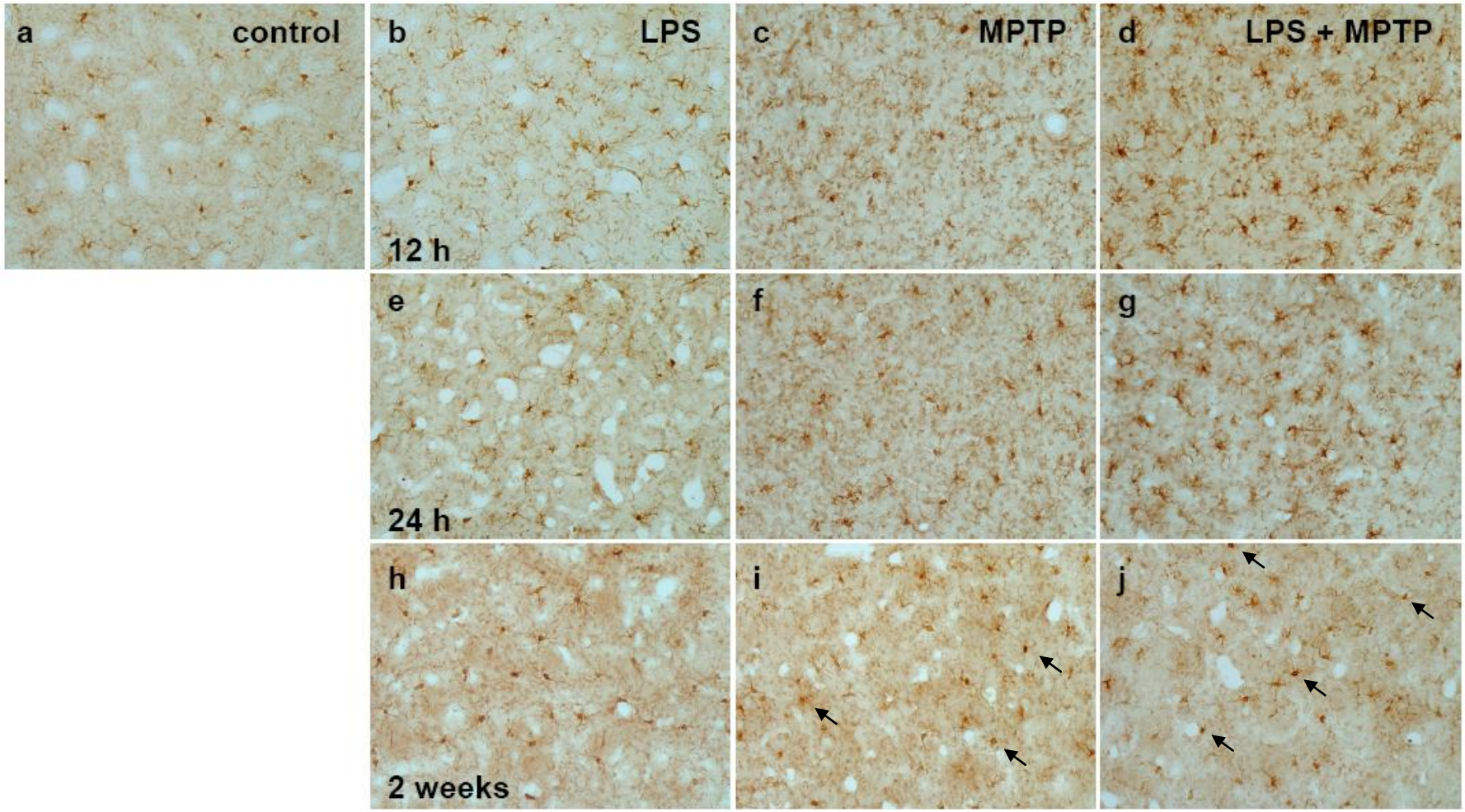

Figure 11. Effect of systemic LPS on MPTP-induced microglia activation in the striatum. Figure shows high magnification (20x lens) photographs of Iba1 immunohistochemistry in the substantia nigra. (a) Control, untreated animals. (b) LPS, 12h. (c) MPTP 12h. (d) LPS + MPTP 12h. (e) LPS 24h. (f) MPTP, 24 h. (g) LPS+MPTP, 24h. (h) LPS, 2 weeks. (i) MPTP, 2 weeks. (j) LPS + MPTP 2 weeks. Arrows show cells with phagocytic morphology.

4.2.2 Substantia nigra

Substantia nigra showed a different time-course of microglial activation when compared with striatal tissue. In animals treated only with MPTP, we failed to detect microglia activation 12 hours after the injection (Figure 12, 13, 14; c). Remarkably, however, the animals treated with both substances still showed stronger activation than animals treated only with LPS. LPS treatment showed increased density compared to controls (144%), LPS+MPTP treatment caused more than double (238%) increase in density of high stained cells (Figure 12, 13, 14; b, d). 24 hours after the treatment, the activation was again similar in LPS treated and MPTP treated animals, and stronger in both substances treated animals (Figures 12, 13, 14; e, f, g). In the high magnification photographs, the activated microglia with irregular shaped bodies and short thick dendrites are visible.

Two weeks after the treatment, the substantia nigra showed results similar to striatum. Microglial morphology did not show clear signs of activation in LPS-only treated animals (Figures 12, 13, 14; h). Most microglia displayed a resting morphology in MPTP treated animals but some cells presented round shape without processes, typical for chronically activated microglia (Figures 12, 13, 14; i). Nigral tissue of LPS + MPTP treated animals showed more phagocytic microglia than MPTP-only treated, and more than in striatal tissue of LPS+MPTP treated (Figures 12, 13, 14; j).

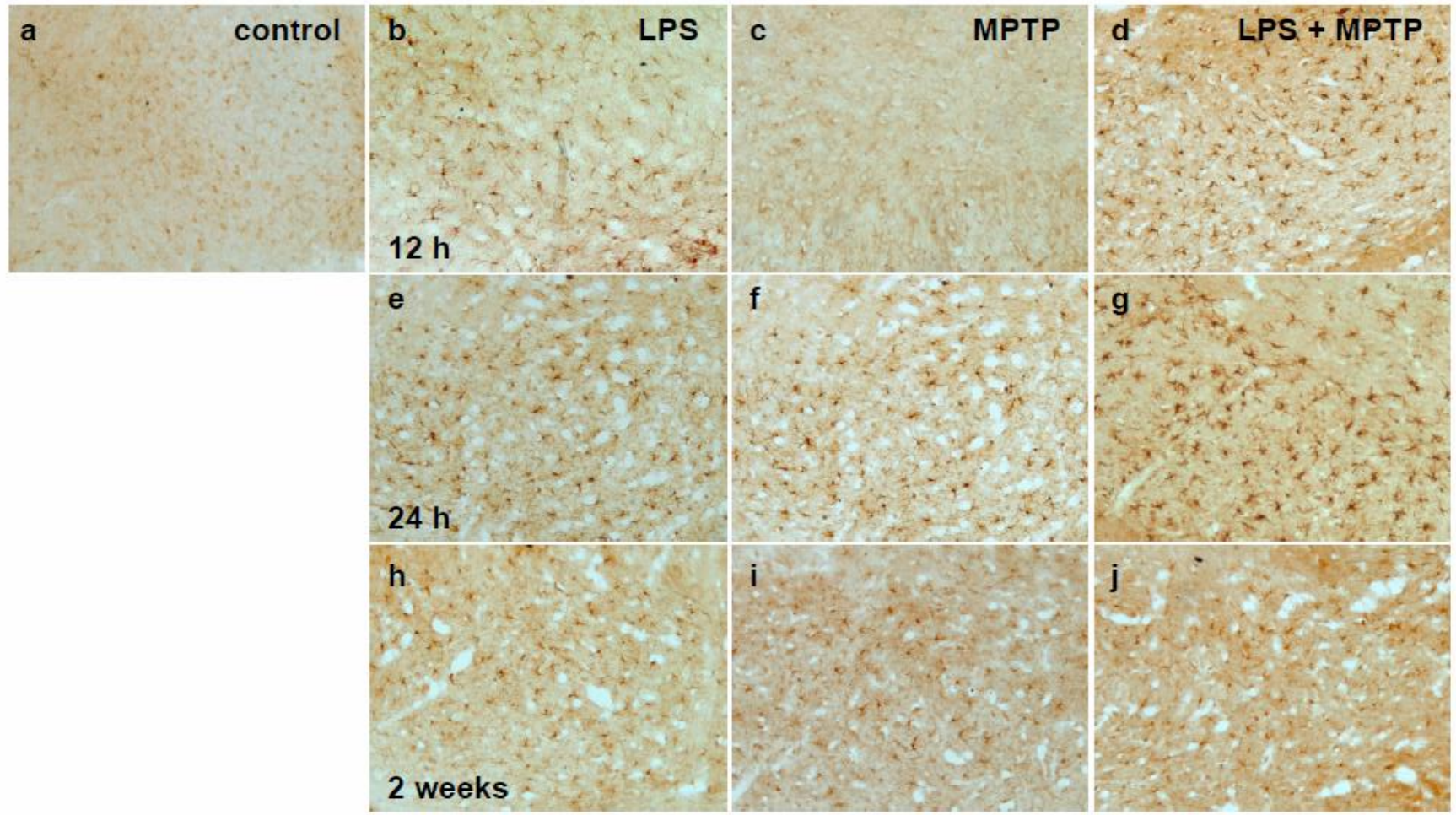

Figure 12. Effect of systemic LPS on MPTP-induced microglia activation in the substantia nigra. Figure shows low magnification (10x lens) photographs of Iba1 immunohistochemistry in the substantia nigra. (a) Control, untreated animals. (b) LPS, 12h. (c) MPTP 12h. (d) LPS + MPTP 12h. (e) LPS 24h. (f) MPTP, 24 h. (g) LPS+MPTP, 24h. (h) LPS, 2 weeks. (i) MPTP, 2 weeks. (j) LPS + MPTP 2 weeks.

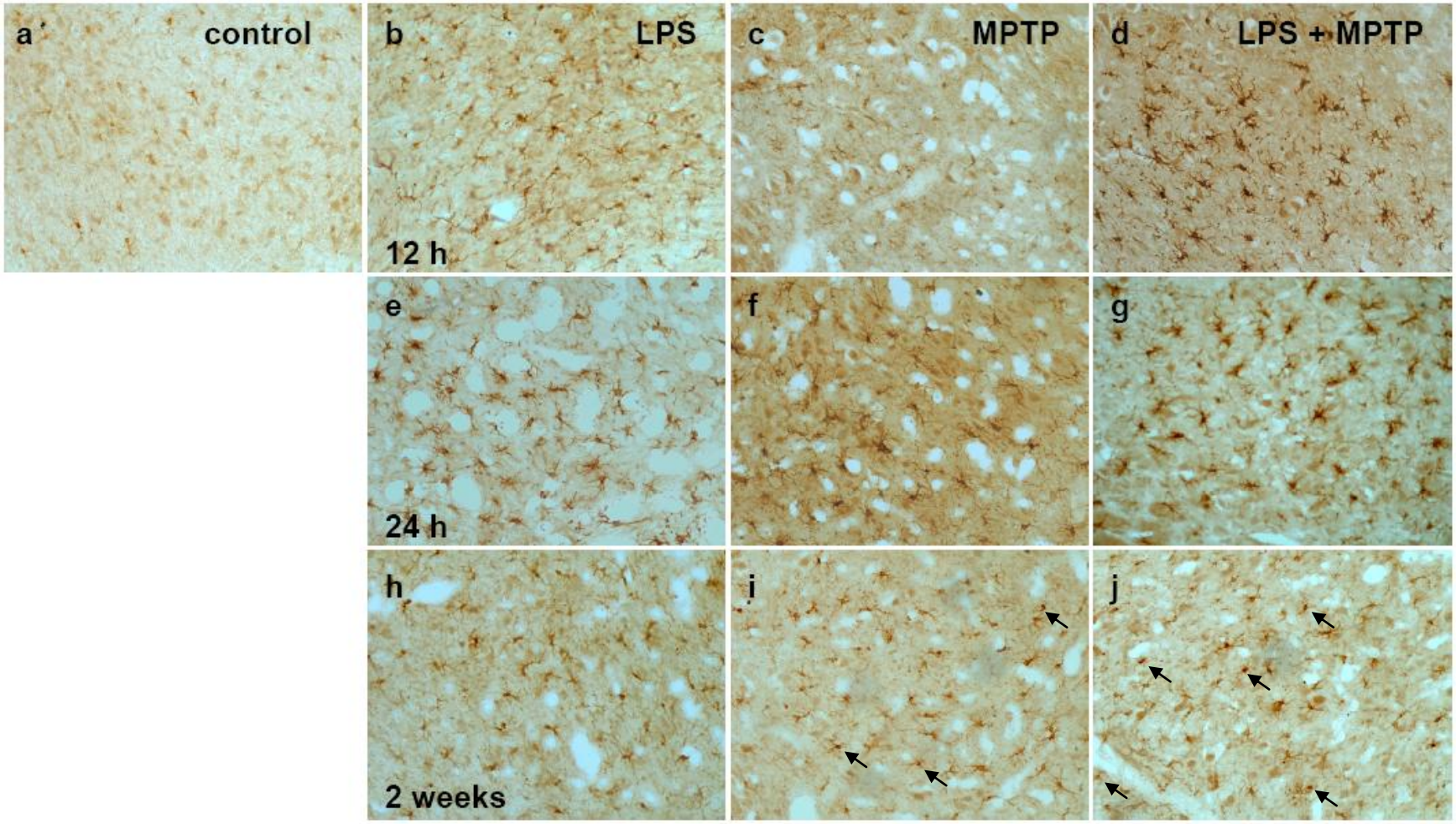

Figure 13. Effect of systemic LPS on MPTP-induced microglia activation in the substantia nigra. Figures show high magnification (20x lens) photographs of Iba1 immunohistochemistry in the substantia nigra. (a) Control, untreated animals. (b) LPS, 12h. (c) MPTP 12h. (d) LPS + MPTP 12h. (e) LPS 24h. (f) MPTP, 24 h. (g) LPS+MPTP, 24h. (h) LPS, 2 weeks. (i) MPTP, 2 weeks. (j) LPS + MPTP 2 weeks. Arrows show cells with phagocytic morphology.

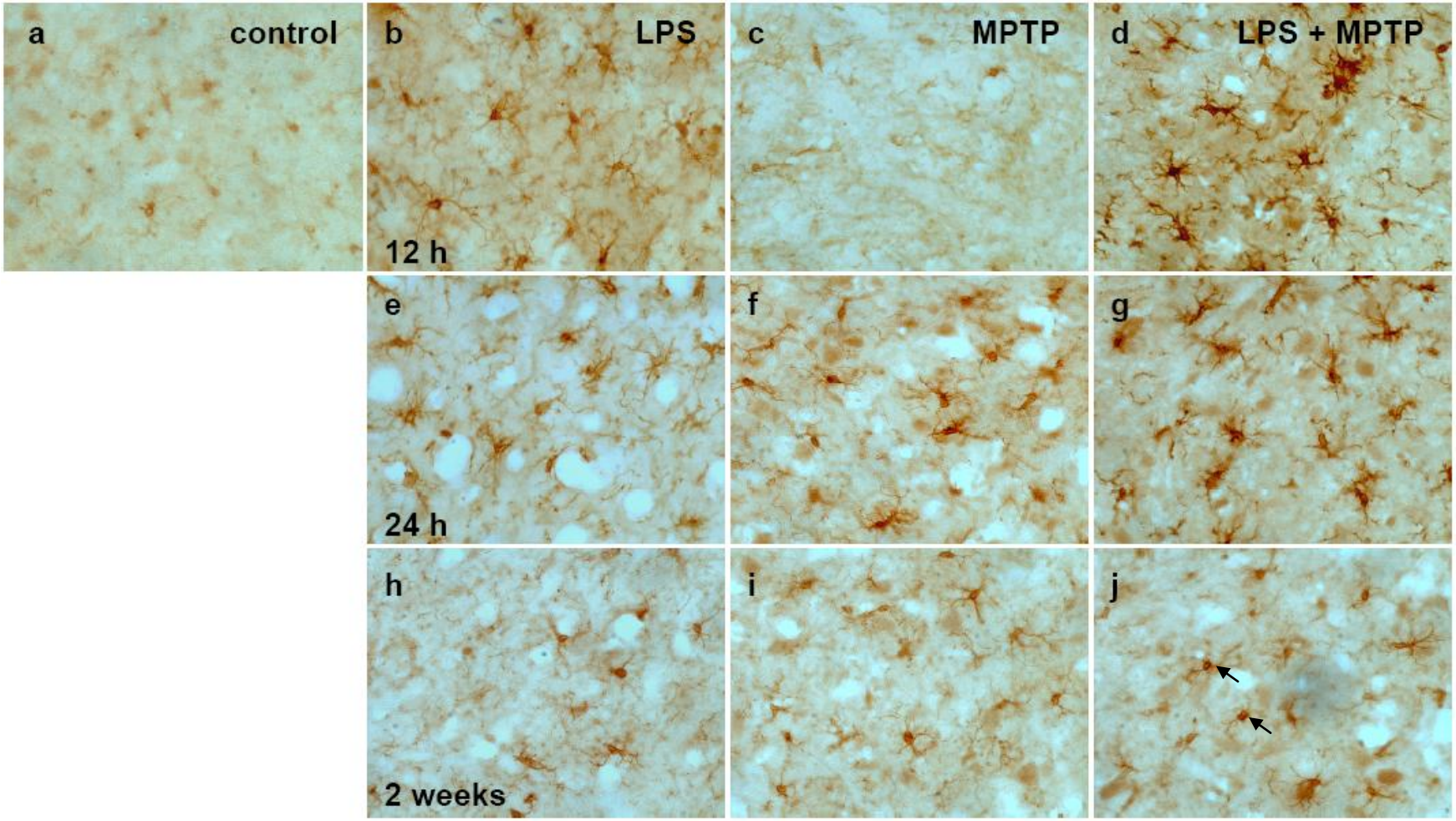

Figure 14. Effect of systemic LPS on MPTP-induced microglia activation in the substantia nigra. Figures show high magnification (40x lens) photographs of Iba1 immunohistochemistry in the substantia nigra. (a) Control, untreated animals. (b) LPS, 12h. (c) MPTP 12h. (d) LPS + MPTP 12h. (e) LPS 24h. (f) MPTP, 24 h. (g) LPS+MPTP, 24h. (h) LPS, 2 weeks. (i) MPTP, 2 weeks. (j) LPS + MPTP 2 weeks. Arrows show cells with phagocytic morphology.

4.3 Blood brain barrier disruption

We studied the disruption of blood brain barrier using immunofluorescence with occludin antibody and immunohistochemistry with IgG antibody.

4.3.1 Occludin

Occludin is a key tight-junction protein critical for the integrity of the blood brain barrier. Upon BBB disruption, occludin expression is down-regulated. We failed to detect an obvious decrease of occludin immunofluorescence in response to either MPTP or LPS+MPTP. Howerver, an atypical pattern of immnofluorescence was observed in the LPS+MPTP-treated animals at 24h (Figure 15, d). Further analysis will be necessary to demonstrate a disruption of the BBB.

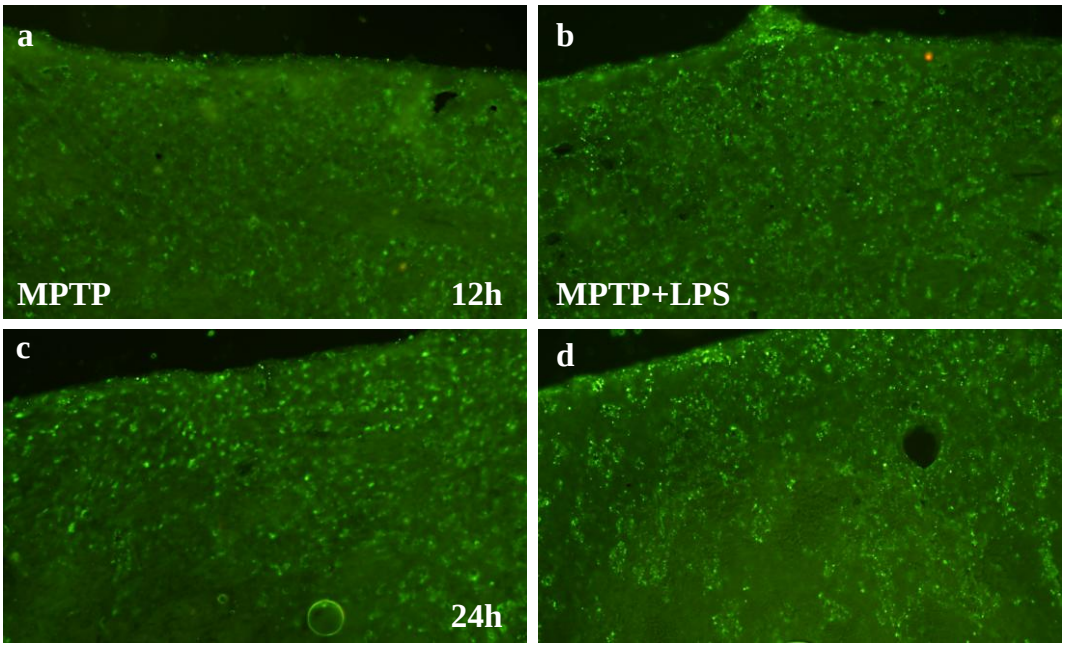

Figure 15. Early time-course occludin expression in striatum in response to either MPTP or LPS+MPTP. Figure shows low-magnification photographs of occludin immunofluorescence in striatum from MPTP (a,c) and LPS+MPTP (b,d) at 12h (a,b) and 24h (c,d). Note the high distribution pattern of occludin expression in the striatal tissue in response to either treatment condition. Also note a patchy-distribution pattern of occluding expression in response to LPS+MPTP 24 h after the treatment.

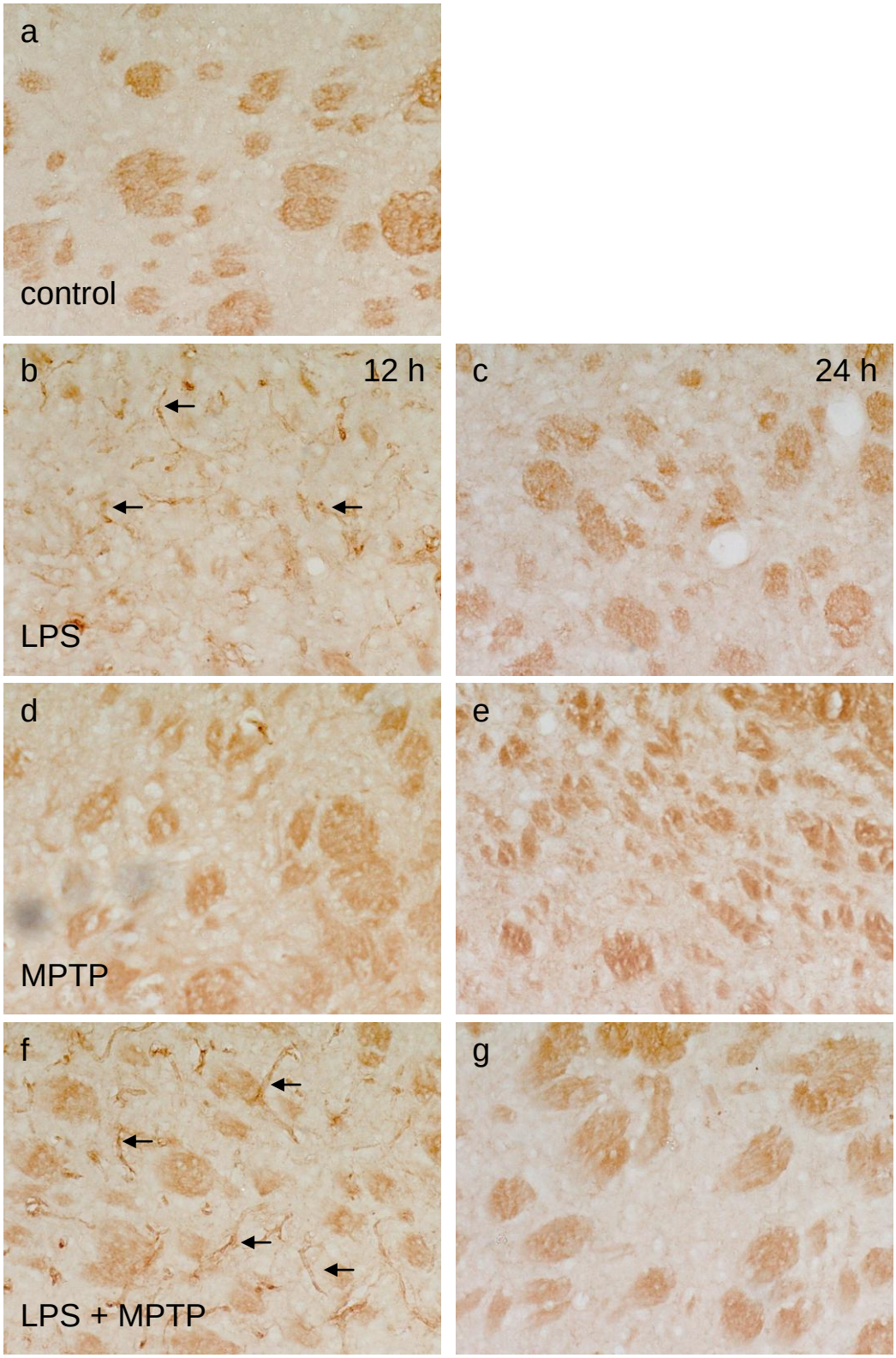

Figure 16. IgG extravasation in striatum in response to systemic LPS and MPTP. (a) Control, without treatment; (c) LPS 24 h; (d) MPTP 12 h; (e) MPTP 24 h; (g)LPS+MPTP 24h. No stained vessels are observable. (b) LPS 12 h; (f) LPS+MPTP 12h. The vessels stained with IgG can be observed (arrows).

4.3.2 IgG

The IgG staining indicates higher vascular permeability 12 hours after the systemic treatment with LPS but not in the brains of MPTP-alone treated animals (Figure 16). This refers to acute BBB disruption after the systemic LPS treatment.

4.4 Levels of dopamine and its metabolites in corpus striatum

To verify the MPTP model of PD, we first studied levels of dopamine and its metabolites in corpus striatum of the mice sacrificed 14 days after the treatment with MPTP, and compared it with the control animals without treatment. Animals receiving single intraperitoneal injection of MPTP showed significant loss in levels of dopamine The average level of dopamine in non treated animals was 26959±3020 ng per gram of the wet tissue, in MPTP treated animals the level dropped to 5718±1448 (Figure 17).

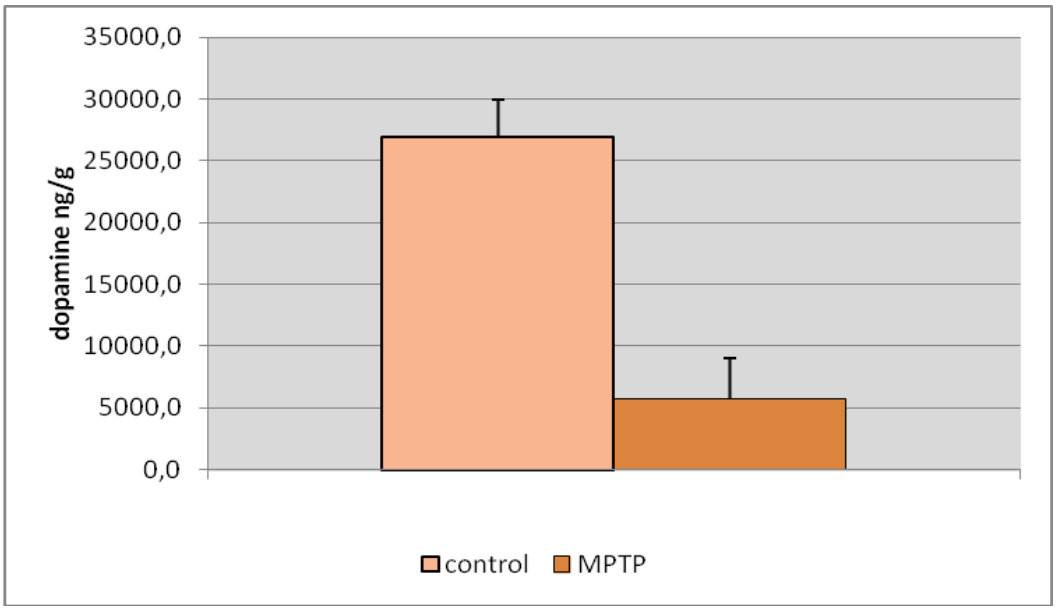

Figure 17. The effect of MPTP on levels of dopamine in the striatum. Levels of dopamine were measured by HPLC and are expressed as ng of dopamine per gram of the wet tissue. Results are mean ± SD of at least 4 animals.

4.5 Dopaminergic neurons loss

We used tyrosine hydroxylase antibody to mark dopaminergic neurons in substantia nigra of mice treated with MPTP or both LPS and MPTP, with the control of animals without any treatment. The control samples showed that the primary antibody was highly selective to dopaminergic neurons. The MPTP treated mice sacrificed two weeks after the treatment showed a significant decrease of the neurons in substantia nigra. The neurodegeneration was stronger in mice treated with both LPS and MPTP. The difference between the neurodegeneration in the LPS+MPTP treated group and group treated with MPTP only was clearly visible. LPS-injected animals failed to induce loss of nigral dopaminergic neurons 2 weeks after the treatment (Figure 18).

As we can see in the figure 19, the stereological analysis confirmed the differences in neurodegeneration in substantia nigra between the group treated with MPTP and the group treated both with MPTP and LPS. Combined treatment of systemic LPS+MPTP exhibited a significant higher loss of nigral dopaminergic neurons as compared with MPTP injected animals. The systemic MPTP produced a loss of TH-immunoreactivity to average 2279±264 TH positive neurons comparing to 5173±1002 in control animals. Combined treatment with LPS and MPTP produced more progressive loss than MPTP only, the avarage number of neurons in SN of mice treated with both substances was 1206±230 (Figure 19).

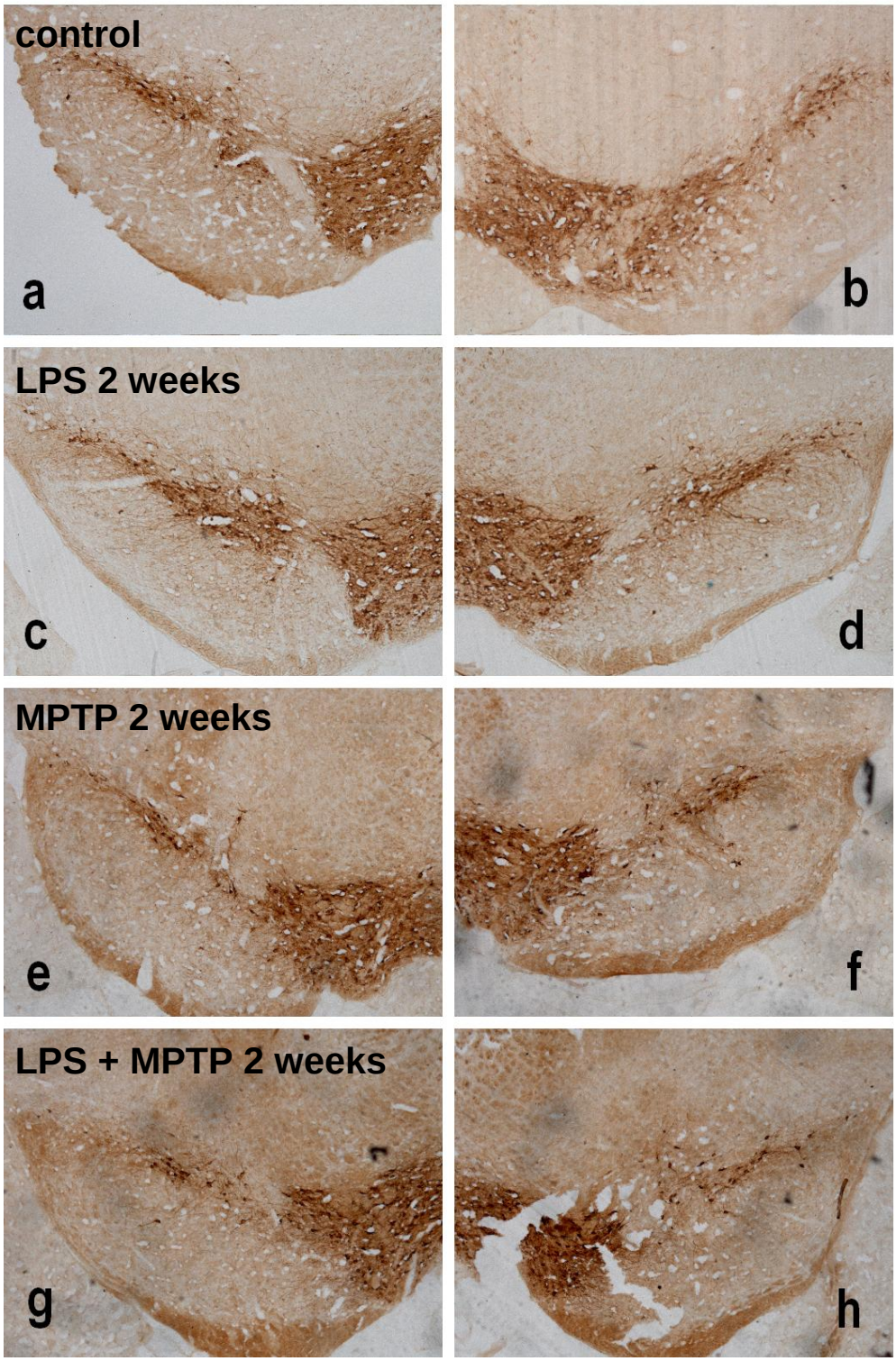

Figure 18. Effect of systemic LPS on MPTP-induced loss of TH immunopositive neurons in the substantia nigra. Figure shows log-magnification photographs (5x lense) of TH immunoreactivity in the ventral mesencephalon of control unlesioned animals (a,b), LPS treated animals (c,d), MPTP treated animals (e,f) and LPS-MPTP treated animals (g,h). Analysis was performed 2 weeks after each experimental condition. Note the significant loss of nigral dopaminergic neurons in response to MPTP (e,f), which was significantly exacerbated in LPS+MPTP (g,h)

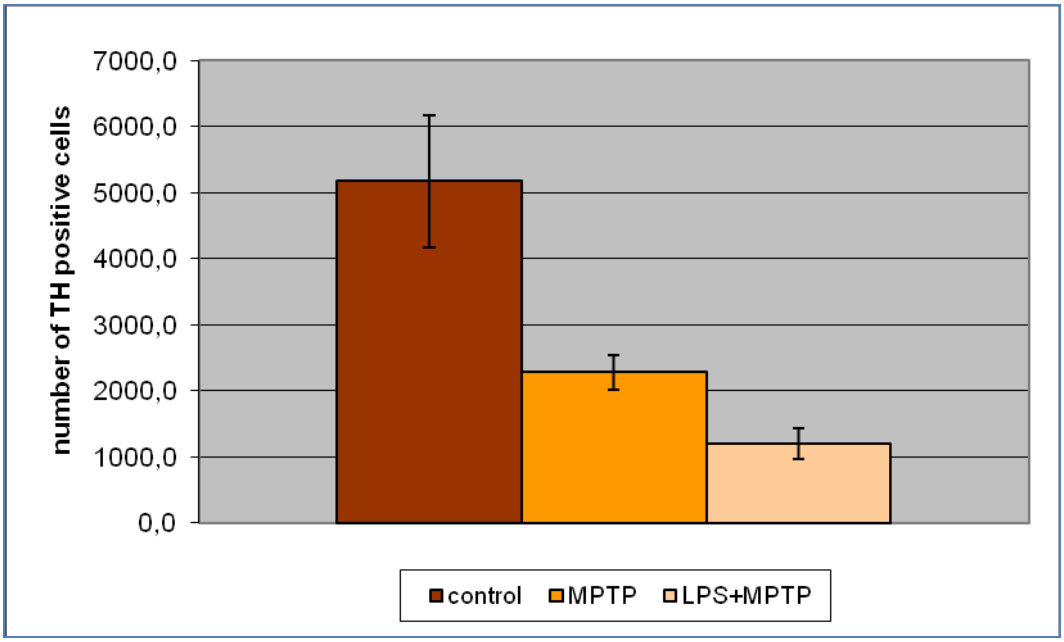

Figure 19. Effect of systemic LPS and MPTP on loss of TH immunopositive neurons in the substantia nigra. The number of TH-immunopositive neurons was quantified for the different treatments by stereological analysis and expressed as number of TH-immunopositive cells per SN. Results are mean ± SD of at least 4 animals.

The loss of TH-immunoreactivity was also observed fourteen days after the treatment with LPS and MPTP in striata of the animals. Figure 20 shows significantly decreased staining in animals treated with MPTP, and very faint staining in striata of animals treated with both LPS and MPTP. 2 weeks after the intraperitoneal injection of MPTP, a significant loss of TH-immunostaining in corpus striatum was observed (Figure 20, g). The striatum of mice treated with both LPS and MPTP showed significantly higher loss of the TH-immunoreactivity (Figure 20, h), comparing to mice treated with MPTP.

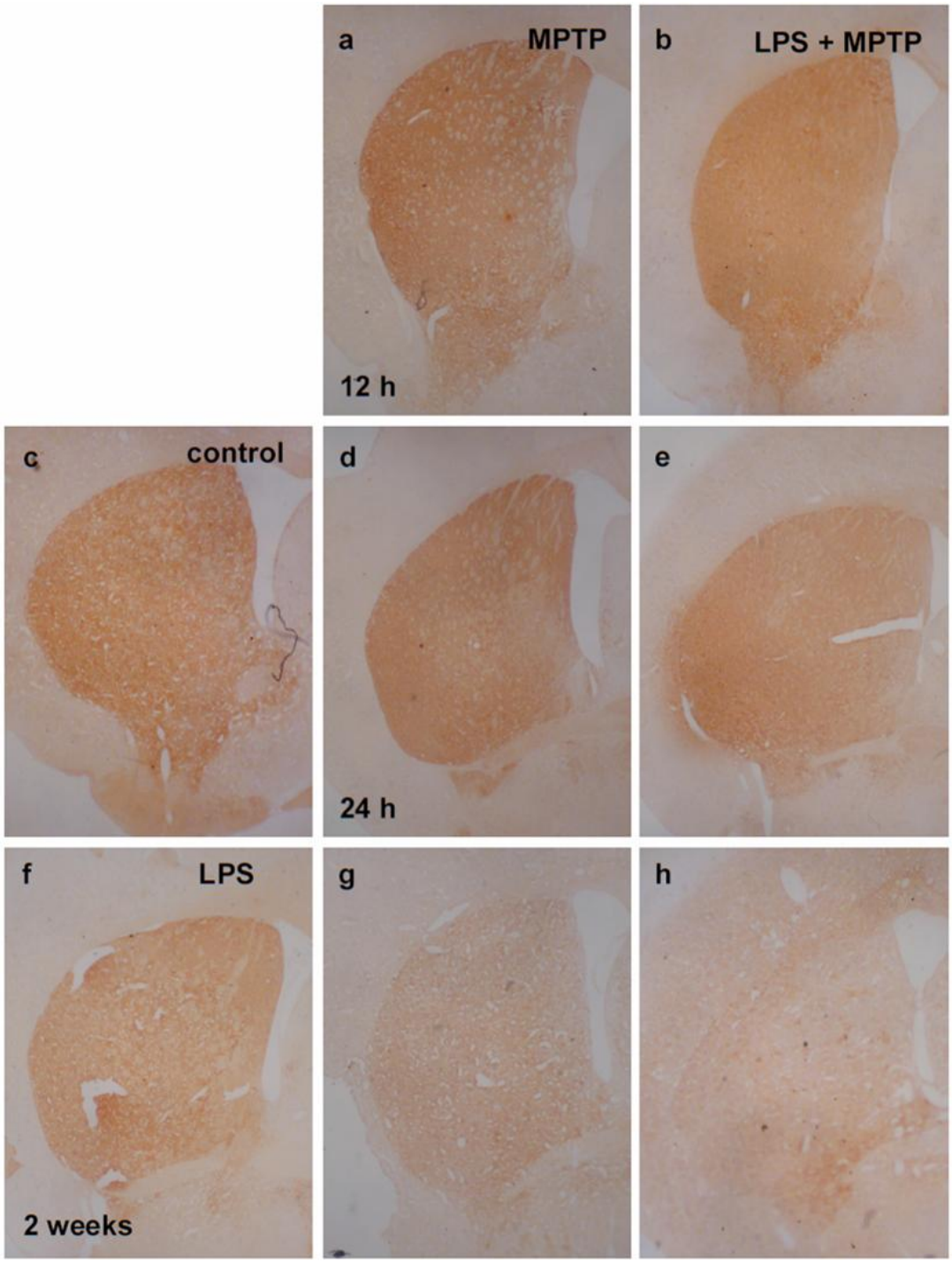

Figure 20. Effect of LPS and MPTP on TH immunoreactivity in corpus striatum. (c) control animals, without treatment. The striatum is marked with brown immunostaining. MPTP 12h (a), LPS+MPTP 12h (b), MPTP 24h (d), LPS+MPTP 24h (e), and LPS 2 weeks (f) show no significant changes in TH-immunoreactivity. MPTP 2 weeks (g) and LPS+MPTP 2 weeks (h) show loss of immunostaining in striatum.

5 DISCUSSION

Our study demonstrates that peripheral inflammation may be a critical factor in exacerbating the brain inflammation and neurodegeneration of the nigrostriatal dopaminergic system in an animal model of PD induced by systemic MPTP administration. This animal model of dopaminergic neurodegeneration was employed for several reasons: i) it induces a specific degeneration of the nigrostriatal dopaminergic system and ii) it fails to alter the integrity of the BBB. Consequently, the MPTP animal model of PD encompasses key features to test the influence of peripheral inflammation in brain neuronal degeneration. Peripheral inflammation was induced by a single systemic dose of LPS. Our data clearly demonstrates the following.

a) Systemic inflammation triggers significantly higher dopaminergic cell death induced by MPTP. This is sustained by a significant greater loss of dopaminergic neurons in the ventral mesencephalon

b) Systemic inflammation clearly makes microglial cells more prone to activation with the subsequent release of different proinflammatory factors, known to play critical roles in neuronal cell death. Overall, combined treatment of LPS-MPTP showed earlier and more intense activation of microglial cells in the nigrostriatal system. Noteworthy, under these conditions, microglia activation took place even earlier than dopaminergic neurodegeneration, a feature compatible with the suggestion that brain inflammation may be a causative factor of dopaminergic neurodegeneration.

The effect of systemic inflammation on neurodegeneration has been already investigated in various animal models. Godoy *et al.* (2008) proved that non-DA-toxic dose of LPS into SN followed by injecting low dose of 6-OHDA increased dopaminergic cell loss when compared to control rats without LPS administration. After injecting an adenovirus expressing a natural endogenous inhibitor of IL-1, IL-1ra, in the SN of rats treated with LPS and 6-OHDA, the neurodegeneration was comparable to that seen in animals treated with 6-OHDA alone. However, under these conditions, the microglial activity was not downregulated. They

further induced systemic inflammation by injecting adenoviral vector expressing IL-1β in the tail vein and found it to exacerbate microglial activation and neurodegeneration in SN induced by either injection of 6-OHDA or adenovirus expressing IL-1β within this structure. This was the first clear description of systemic inflammation exacerbating DA neurodegeneration in SN caused by an insult, and these studies raised an important role of IL-1β in mediating brain inflammation and associated neurotoxicity.

Villarán *et al.* (2010) used a model of peripheral inflammation associated to ulcerative colitis induced by dextran sodium sulphate administration to study effect of systemic inflammation in the LPS intranigral injection model of PD. They demonstrated that under these conditions, increased inflammation was found in SN along with alterations in BBB and higher DA neuron loss.

However, it should be noted that all these studies used invasive methods to induce the neurodegeneration in SN. Injection of 6-OHDA , LPS or vector expressing IL-1 right into the SN causes a clear disruption of the BBB (Tomás-Camaridel *et al.* 2005), and this effect itself can fundamentally influence the results of experiments on systemic inflammation. As described above, BBB plays an essential role in communication between systemic inflammation and brain, and its breakdown causes massive infiltration of peripheral cells and brain toxic substances into the brain. This is a critical condition that prevent from drawing any conclusion about the role of peripheral inflammation in dopaminergic neurodegeration from these models.

In our experiments, we use MPTP, a lipophilic molecule that, administered systemically, crosses the BBB without damaging it, and induces key parkinsonian-like changes in the brain. Hence, it provides a better model for studying the effects of systemic inflammation on PD.

Interesting insights brought some experiments on effects of peripheral inflammation on some other neurodegenerative disorders. Lunnon *et al.* (2011) pointed out the essential role of activated microglia in the pathogenesis of prion disease. Systemic LPS challenge resulted in aggressive proinflammatory response of microglia, leading to significantly faster progression of the disease.

Systemic inflammation in form of intraperitoneal injection of LPS was also reported to potentiate acute phase of stroke and exacerbate damage of the brain in experimental mouse model of stroke. This effect was abolished by administration of IL-1 receptor antagonist, and systemic IL-1 administration had similar detrimental impact as LPS, pointing out importance of IL-1 as a critical mediator. Another important role took neutrophils, as the damaging effects of systemic IL-1 were abolished by neutropenia (McColl *et al.* 2007). These experiments point out the importance of BBB integrity. It should be mentioned the importance of neutrophil-derived matrix metalloproteinase-9 (MMP9), which was five-fold increased in mice with systemic IL-1β challenge (McColl *et al.* 2008). This led to increase in neurovascular gelatinolytic activity, which altered the kinetic of BBB disruption. Stroke leads to acute disruption of tight junction protein claudin-5, but systemic inflammation converted this disruption to sustained, which has been indicated as a mechanism underlying the exacerbation of stroke by systemic inflammation (McColl *et al.* 2008).

Consequently, experimental data highlights the importance of systemic inflammation in exacerbating impairment of the BBB and neurodegeneration, especially in acute inflammatory conditions.

There is increasing evidence about the role of BBB integrity in PD ethiology. Increased vascular permeability has been reported in PD patients (Popescu *et al.* 2009, Kortekaas *et al.* 2005). In addition, alterations of BBB transport has been reported in aged, non PD individuals. Hence, a single systemic inflammation could exacerbate slight alterations in BBB transport and possibly lead to persisting neuroinflammation and neurodegeneration. Our data show that systemic inflammation triggers alterations in BBB, and we proved that systemic inflammation, in absence of other BBB damaging insult, exacerbates the neurodegeneration in the MPTP model of PD. Taken together, these results outline the important role of BBB in the implication of systemic inflammation in PD pathogenesis. However, further studies have to be carried out to better understand the molecular and cellular mechanisms. Thus, we aim to determine cell infiltration of T-lymphocytes, granulocytes and peripheral macrophages in the nigrostriatal dopaminergic system in our LPS+MPTP model, to evaluate the role

of these infiltrated cells in the progression of the persisting neuroinflammation and neurodegeneration.

Interesting results brought a study on prenatal exposure to LPS, resulting in higher levels of TNFα and increased susceptibility to 6-OHDA in adulthood (Ling *et al*. 2004). Beside the fact that this study again uses intranigral 6-OHDA model of PD, in which BBB is damaged and the communication between systemic inflammation and brain is different from that in PD pathology, it refers to the involvement of cytokines, in particular TNFα, in PD progression.

As mentioned above, the levels of cytokines are elevated in PD patients as well as in all the PD animal models. Interestingly, they have been reported as a possible causative factors. Studies on promoter region polymorphisms in the TNFα gene in cohort of 172 Japanese patients with sporadic PD showed that frequency of the -1031C allele, a high producer of TNF, was significantly higher in early onset PD patients compared to late onset PD patients and unaffected controls (Nishimura *et al.* 2001). TNFα is highly toxic to dopaminergic neurons, as has been described in many studies (McCoy and Tansey, 2008). Thus, evidence that systemic inflammation could trigger long-lasting higher levels of cytokines in brain would bring important insight to their role in PD initiation and progression.

Our results showed that systemic LPS pre-treatment increases mRNA expression of IL-6 in striatum and substantia nigra in MPTP treated animals. mRNA expression of TNFα was higher in substantia nigra, but not in striatum of LPS+MPTP treated animals, compared to MPTP injected animals, 24 hours after the treatment. We suggest that this difference between IL-6 and TNFα could be caused by the different time-course in the response to the inflammatory stimulus. Furthermore, differences in mRNA expression of TNFα between SN and striatum could be result of the time-course of the responsiveness of SN and striatum. TNFα expression in the midbrain after systemic LPS administration peaks at 8 hours after the LPS insult (Jeong *et al*. 2010), and the time course of its elevation varies in different brain structures including the striatum, in which the elevation is very fast. Thus, we suggest that in our experimental conditions, TNFα mRNA expression is faster in striatum than in substantia nigra. The same could be true in LPS pre-treated animals. Supporting this view, we observed an earlier microglial

activation in striatum in LPS+MPTP animals as compared to MPTP-treated mice. Moreover, microglial activation in striatum preceded their activation in SN, a view compatible with the view that striatum is the main target tissue of MPP+, the active toxic metabolite of MPTP. In addition, the expression of mRNA encoding for iNOS was lower in LPS+MPTP-treated animals compared to MPTP injected mice 24 hours after the insult. Overall LPS+MPTP-treated animals showed higher brain inflammation, as characterized by higher microglial activation and increased cytokine production. Consequently, lower levels of iNOS in LPS+MPTP treated mice is a puzzling result. However, an earlier response of iNOS in these animals could refer to the reported differences. Additional experiments are required to shed light on this issue, for example western blot analysis of iNOS in the nigrostriatal system under different experimental conditions.

There is a report that strong peripheral inflammation alone may cause DA neuronal loss. High dose of LPS (5mg/kg) administered intraperitonealy to mice caused inflammation in the brain resulting in progressive neurodegeneration. The loss of TH-immunoreactive neurons was measured 7 and 10 months after the treatment and was 23% and 47%. The mechanism was explained as follows: synthesis of TNFα in brain was induced by liver-derived TNFα (the immediate response to systemic LPS), and while the levels of blood TNFα decreased rapidly to normal, in the brain the protein remained elevated even 10 months after the treatment. TNFα receptors were suggested to be essential for transport of this protein across the BBB. In the brain, TNFα was reported to induce synthesis of other proinflammatory factors and trigger sustained activation of microglia (Qin *et al*. 2007). Limitation of this study is that this huge dose of LPS caused a condition close to septic shock. As authors proposed, it could have been interesting to study some link between septic shock and incidence of PD.

In another study, LPS alone (in a dose of approximately 2mg/kg) was reported to cause brain inflammation but not significant DA neuronal death. The LPS was administred to rats through tail vein. Rapid inflammatory response in brain (within 8 hours) and infiltration of neutrophils as well as activation of microglia were reported, but no dopaminergic neuronal loss, counted as number of TH-immunoreactive neurons in SN 8 days after the treatment, was evident.

Expression of TNFα and other cytokines in SN peaked 8 hours after the LPS administration and then decreased close to the basal levels (Jeong *et al.* 2010). These results are in agreement with our findings, showing that systemic LPS causes inflammation in the brain, but fails to induce dopaminergic neurodegeneration This, however, does not exclude the possible role of systemic inflammation as an insult sufficient to cause neurodegeneration in aged or other way already damaged brain.

Very interesting results reported studies on systemic LPS in aged mice. The inflammatory response was found amplified and prolonged in aged brain comparing to young individuals, and behavioral deficits induced by LPS were more permanent in the aged. It is worthy mentioning that elderly are more susceptible to infections, because aging is associated with deteriorated function of immune system (Godbout *et al.* 2005). This could raise a question whether systemic inflammation, even if in healthy young brain does not cause permanent neuroinflammation, could be in aged brain sufficient to produce persisting microglial activation, leading to neurodegeneration.

6 CONCLUSION

Our study demonstrates that LPS-induced acute systemic inflammation triggers rapid activation of microglia in striatum and substantia nigra. Very interesting, peripheral inflammation significantly enhances the microglial activation caused by MPTP in mice, one of the best animal models of PD, and more important, it induces a higher dopaminergic neurodegeneration in the nigrostriatal system. Our data supports the notion that the long-lasting higher microglia activation induced by peripheral inflammation may play a significant role DA neurodegenerative process, the most relevant hallmark of PD. Systemic inflammation stimulus by increasing acute BBB permeability, striatal expression of IL-6 and nigral expression of TNF-α may sensitize nigral dopaminergic neurons to further deleterious stimulus.

We conclude that:

(i) Acute systemic inflammation alone triggers brain inflammation in areas typically related to PD in absence of other brain deleterious stimulus, including the corpus striatum and substantia nigra.

(ii) Acute systemic inflammation enhances and accelerates the microglial activation caused by MPTP in absence of other BBB damaging agents

(iii) Acute systemic inflammation synergistically interact with MPP+, the toxic metabolite of MPTP, to cause higher death of dopaminergic neurons in the substantia nigra.

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8 ABBREVIATIONS

PD – Parkinson´s Disease

DA – dopamine

SN – Substantia nigra

LBs - Lewy bodies

LNs – Lewy neurites

AD – Alzheimer´s disease

LRRK-2 - leucin-rich repeat kinase 2

LPS – lipopolysaccharide

IL-6 – interleukin 6

IL-1 – interleukin 1

IFNγ – interferon gamma

COX2 – cyclooxygenase 2

TNFα – tumour necrosis factor alpha

NSAIDs - non-steroidal anti-inflammatory drugs

PGE_2 - prostaglandin E_2

BBB - blood-brain barrier

CNS – central nervous system

MMP3 - matrix metalloproteinase 3

BDNF - brain-derived neurotrophic factor

GDNF - glial cell line-derived neurotrophic factor

SNc – substantia nigra, pars compacta

Nrf2 - nuclear factor erythroid 2-related factor 2

ARE - antioxidant response element

ROS - reactive oxidative species

PET - positron emission tomography

VEGF – vascular endothelial growth factor

CD4 – cluster of differentiation 4

Iba1 - ionized calcium binding adaptor molecule 1

RNS - reactive nitrogen species

iNOS - inducible NO synthese

MAO - monoamine oxidase

DOPAC - dihydrophenylacetic acid

TLR - toll-like receptors

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

CD14 - cluster of differentiation 14

MPTP - *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

PBS - phosphate buffered saline

HPLC - high-performance liquid chromatography

qPCR – quantitative polymerase chain reaction

RT-PCR – real time polymerase chain reaction

TBS – tris buffered saline

TH – tyrosine hydroxylase